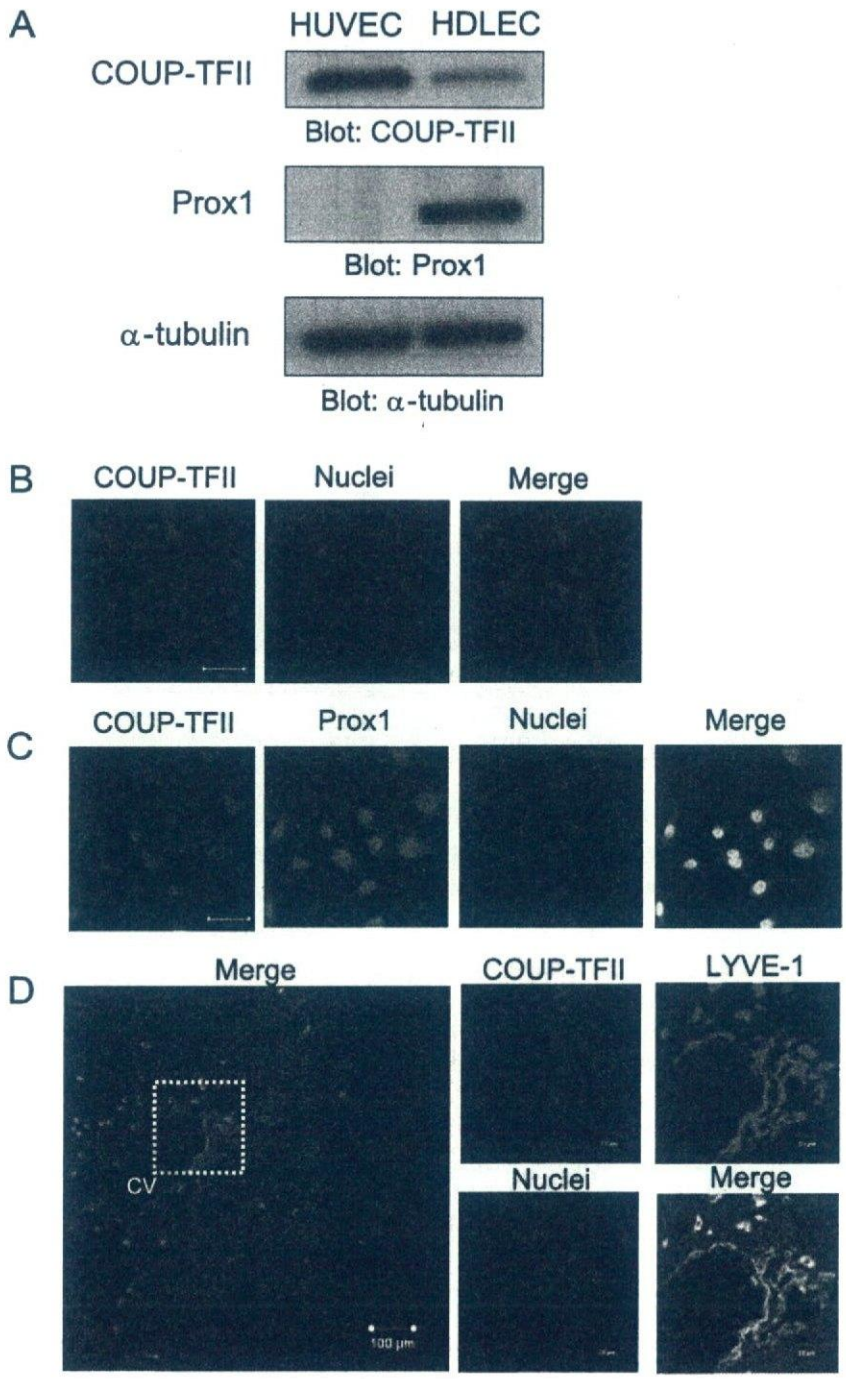

Figure 1 COUP-TFII is expressed in BECs and LECs. (A) Western blot analyses for COUP-TFII (top panel) and Prox1 expressions (middle panel) in HUVECs and in HDLECs. α-tubulin was used as a loading control (bottom panel). (B–C) Immunostaining of HUVECs (B) and HDLECs (C) was carried out for COUP-TFII (red) and Prox1 (green) with nuclear staining by TOTO3 (blue). COUP-TFII and Prox1 were co-localized to nuclei in HDLECs (see Merge). Scale bars, 50 μm. (D) Immunohistochemistry was carried out for COUP-TFII (red) and LYVE-1 (green) with nuclear staining by TOTO-3 (blue) using transverse sections at the level of the heart of 11.5 dpc mouse embryo. Right small panels are magnified images of the boxed area of the left large panel. CV; cardinal vein. Scale bars, 100 μm (left panel) and 20 μm (right four panels).

We also observed that COUP-TFII protein was localized to the nuclei of HUVECs (Fig. 1B) and co-localized with Prox1 in HDLECs (Fig. 1C). The specificity of anti-COUP-TFII antibody used was confirmed using the HUVECs whose expression of COUP-TFII was knocked down by siRNA (Fig. S1 in Supporting Information).

Furthermore, we examined the COUP-TFII expression in embryos. At 11.5 dpc of mouse development, LECs that are positive for LYVE-1, a LEC marker, sprout out from cardinal veins (Fig. 1D) (Oliver 2004). We observed that these sprouting LECs express COUP-TFII (Fig. 1D). These results suggest that COUP-TFII is temporally and spatially co-localized with Prox1 in LECs.

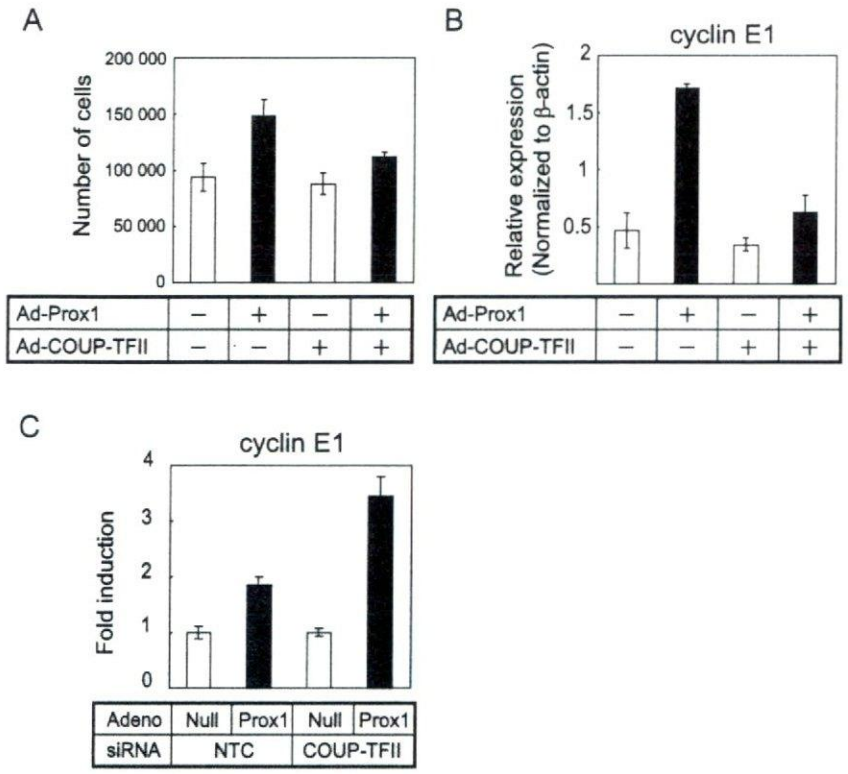

Figure 2 COUP-TFII suppresses Prox1-induced cell proliferation through the regulation of cyclin E1. (A) Numbers of cells were counted 48 h after infection of HUVECs with adenovirus coding for Prox1 (Ad-Prox1) in combination with that for COUP-TFII (Ad-COUP-TFII). Each value represents the mean of triplicate determinations; Bars, SD. (B–C) Effects of gain- and loss-of-function of COUP-TFII on the expression of cyclin E1 in HUVECs. HUVECs were infected with Ad-Prox1 in combination with Ad-COUP-TFII (B) or siRNAs for COUP-TFII (C), followed by quantitative RT-PCR analysis for cyclin E1. Non-coding adenovirus (Null) and siRNA carrying scrambled sequences (NTC) were used as negative controls. Bars, SD.

COUP-TFII suppresses Prox1-induced cell proliferation through the regulation of cyclin E1 expression

While Prox1 was reported to induce cyclin E1 expression in HDMECs (Petrova *et al.* 2002), its effects on endothelial cell proliferation have not yet been examined. When Prox1 was expressed in HUVECs using adenovirus, it significantly increased cell number (Fig. 2A). In order to examine the effect of COUP-TFII on Prox1-mediated promotion of endothelial cell proliferation, we increased the level of COUP-TFII expression by adenovirus coding for COUP-TFII. While COUP-TFII expression itself did not affect cell proliferation, elevated cell proliferation by Prox1 was significantly repressed by COUP-TFII (Fig. 2A).

In order to dissect the molecular mechanisms, we carried out quantitative RT-PCR analysis for cyclin E1 (Fig. 2B) and E2 (Fig. S2 in Supporting Information). In accordance with the result of cell proliferation, COUP-TFII significantly suppressed the cyclin E1 expression induced by Prox1 (Fig. 2B), which was also confirmed for cyclin E2 expression (Fig. S2A in Supporting Information).

We next examined whether endogenous COUP-TFII is necessary to suppress Prox1-mediated induction of cyclin E1 expression by knocking down endogenous COUP-TFII expression using siRNA (Fig. S1 in Supporting Information). As shown in Fig. 2C, the induction of cyclin E1 expression by Prox1 was significantly increased by the loss of COUP-TFII expression, which was also confirmed for cyclin E2 expression (Fig. S2B in Supporting Information). These findings suggest that COUP-TFII suppresses Prox1-induced cell proliferation by interfering with cyclin E expression.

COUP-TFII suppresses Prox1-mediated endothelial cell migration towards VEGF-C by regulating VEGFR3 expression

We recently showed that Prox1 induces endothelial cell migration towards VEGF-C through up-regulation of VEGFR3 expression (Mishima *et al.* 2007). To examine the effect of COUP-TFII on the Prox1-mediated promotion of endothelial chemotaxis towards VEGF-C, we carried out chamber migration assays using HUVECs. As shown in Fig. 3A, COUP-TFII significantly suppressed the chemotaxis towards VEGF-C enhanced by Prox1.

In consistent with the results of the chamber migration assay, COUP-TFII suppressed the VEGFR3 mRNA expression induced by Prox1 (Fig. 3B). This result was further confirmed at protein level (Fig. 3C). In addition,

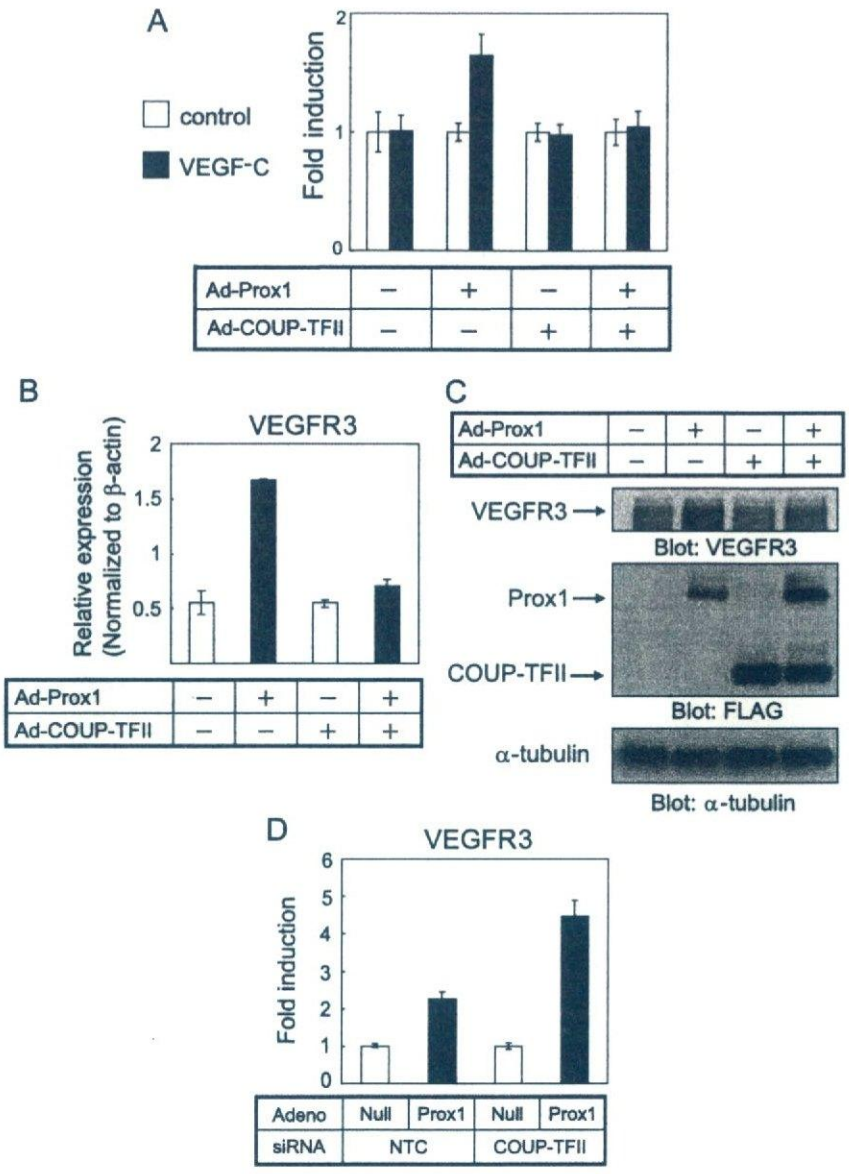

Figure 3 COUP-TFII represses Prox1-induced endothelial cell migration towards VEGF-C via regulation of VEGFR3 expression. (A) Effect of COUP-TFII on the Prox1-induced endothelial cell migration towards VEGF-C. Migration of HUVECs infected with indicated adnoviruses was measured by Boyden chamber assay as described previously (Mishima *et al.* 2007). Relative migration towards VEGF-C is shown as a ratio of the number of migrated cells in the presence of VEGF-C against that in the absence of VEGF-C. Bars, SD. (B–C) Effect of gain-of-function of COUP-TFII on the Prox1-induced expression of VEGFR3. HUVECs were infected with indicated adenoviruses, followed by quantitative RT-PCR (B) and Western blotting (C: top panel) for VEGFR3. Western blotting was also carried out for FLAG-tagged Prox1 and COUP-TFII transduced by adenoviruses (middle panel). α-tubulin was used as a loading control (bottom panel). (D) Effect of loss-of-function of COUP-TFII on the Prox1-induced expression of VEGFR3. HUVECs were infected with adenovirus coding for Prox1 (Ad-Prox1) or non-conding adenovirus (Ad-Null) in combination with siRNA for COUP-TFII or negative control siRNA (NTC), followed by quantitative RT-PCR for VEGFR3. Bars, SD.

when COUP-TFII expression was knocked down in HUVECs, induction of VEGFR3 expression by Prox1 was enhanced (Fig. 3D). These results suggest that COUP-TFII suppresses Prox1-induced endothelial chemotaxis towards VEGF-C by regulating VEGFR3 expression.

Endogenous level of COUP-TFII expression in HDLECs is required to maintain the expression of VEGFR3

We next investigated the roles of COUP-TFII in the LECs in which endogenous Prox1 is expressed. When the level of COUP-TFII expression was elevated in HDLECs, both proliferation (Fig. 4A) and chemotaxis towards VEGF-C (Fig. 4C) were suppressed with concomitant decrease in the expression of cyclin E1 (Fig. 4B) and VEGFR3 (Fig. 4D). These findings were consistent with the results observed in HUVECs. However, when COUP-TFII was knocked down in HDLECs, their migration towards VEGF-C and VEGFR3 expression were attenuated (Fig. 4E,F), whereas neither cell number nor cyclin E1 expression changed (data not shown). These results suggest that COUP-TFII differentially functions between HDLECs and HUVECs.

We further examined whether COUP-TFII alters the endogenous Prox1 expression in LECs. As shown in Fig. 4G, endogenous Prox1 expression was significantly decreased when the level of COUP-TFII expression was

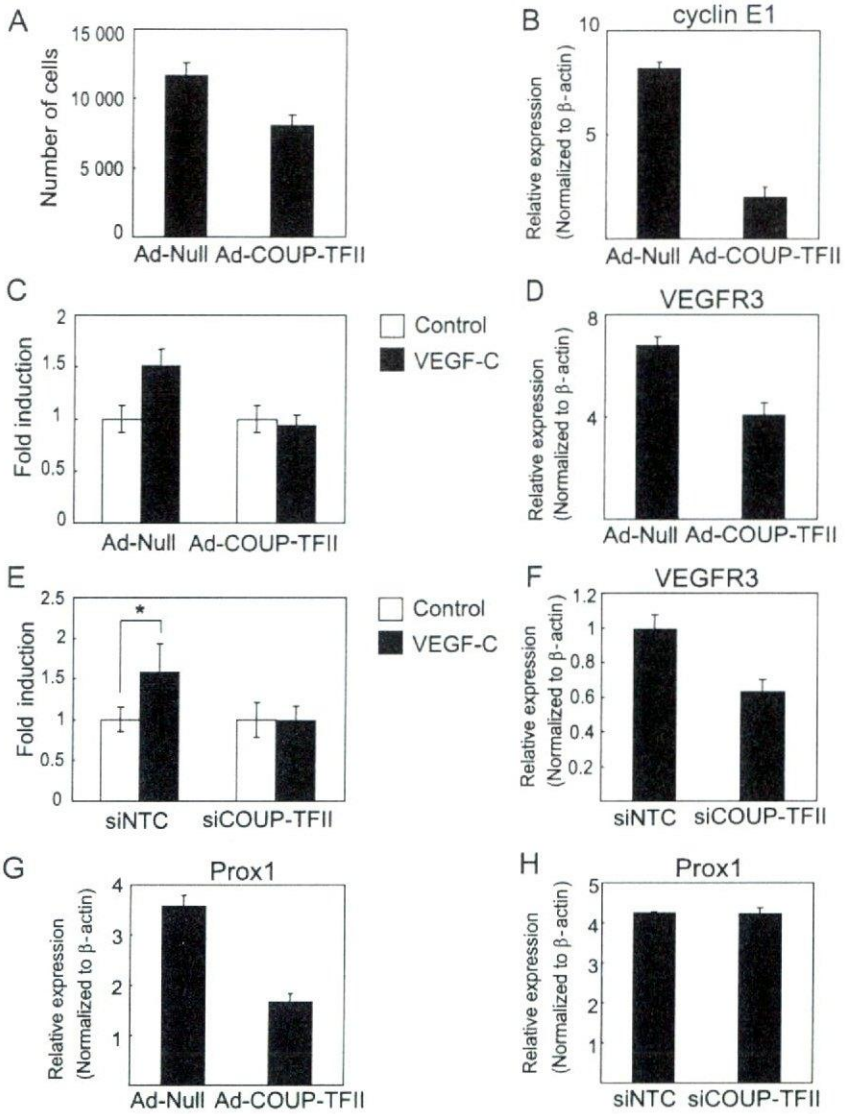

Figure 4 Endogenous level of COUP-TFII expression plays important roles in the maintenance of characteristics of HDLECs. (A–D) HDLECs infected with non-coding adenovirus (Ad-Null) or adenovirus coding for COUP-TFII (Ad-COUP-TFII) were subjected to cell proliferation assay (A), chamber migration assay using VEGF-C as an attractant (C) and quantitative RT-PCR analyses for cyclin E1 (B) and VEGFR3 (D). (E, F) HDLECs transfected with control siRNA (siNTC) or siRNA for COUP-TFII (siCOUP-TFII) were subjected to chamber migration assay using VEGF-C as an attractant (E) and quantitative RT-PCR analyses for VEGFR3 (F). In (C) and (E), relative migration towards VEGF-C is shown as a ratio of the number of migrated cells in the presence of VEGF-C against that in the absence of VEGF-C. (G, H) Expression of endogenous Prox1 expression was determined in the HDLECs infected with Ad-COUP-TFII (G) or transfected with siCOUP-TFII (H). $*P < 0.01$. Bars, SD.

elevated in HDLECs, while the loss of COUP-TFII expression did not alter the Prox1 expression (Fig. 4H). These results suggest that excessive level of COUP-TFII may interfere with the functions of Prox1 directly by inhibiting the transcriptional activities of Prox1 and indirectly by suppressing the endogenous Prox1 expression.

COUP-TFII interacts with Prox1 in HDLECs

Previous reports that LRH-1 and SF-1, members of nuclear receptor superfamily, bind Prox1 (Qin *et al.* 2004; Steffensen *et al.* 2004) prompted us to examine whether COUP-TFII and Prox1 interact. We carried out co-immunoprecipitation experiments with cell lysates prepared from the HUVECs infected with adenoviruses coding for Prox1 and COUP-TFII (Fig. 5A). When the lysates were subjected to immunoprecipitation, we detected COUP-TFII in the immunoprecipitates pulled down with anti-Prox1 antibody, which indicates that Prox1 is capable of interacting with COUP-TFII in HUVECs.

Next, we examined whether endogenous Prox1 and COUP-TFII interact in HDLECs using the Duolink *in situ* proximity ligation assay (PLA). This method enables us to monitor subcellular localization of endogenous protein–protein interactions at single molecule resolution (Söderberg *et al.* 2006, 2008). In the HUVECs infected with adenoviruses coding for Prox1 and COUP-TFII, we detected a number of strong fluorescence signals in the presence of specific antibodies, which indicates the interaction between Prox1 and COUP-TFII in the

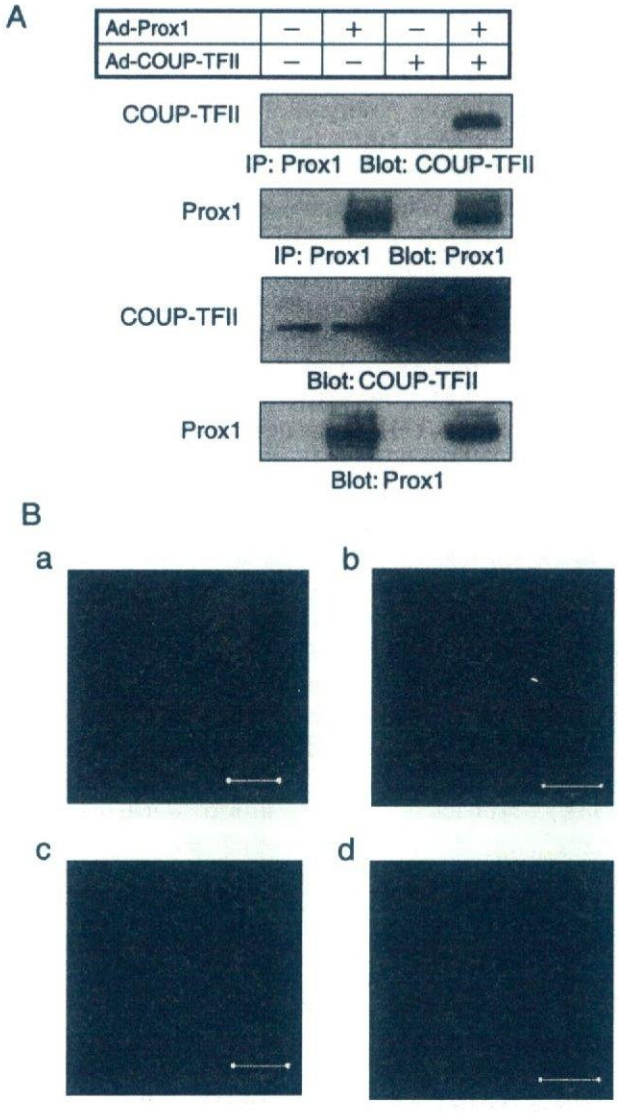

Figure 5 COUP-TFII physically interacts with Prox1. (A) Co-immunoprecipitation of COUP-TFII with Prox1 in HUVECs. HUVECs infected with adenovituses coding for Prox1 (Ad-Prox1) and COUP-TFII (Ad-COUP-TFII) were subjected to immunoprecipitation with anti-Prox1 antibody, followed by immunoblotting with anti-COUP-TFII antibody (top panel). Expression of Prox1 and COUP-TFII was also confirmed. (B) Interaction of endogenous Prox1 and COUP-TFII in HDLECs. PLA was carried out to detect the proximal location of Prox1 and COUP-TFII (shown as red dots) as described in Experimental Procedures. All samples were counterstained with TOTO3 (blue) to visualize nuclei. (a–c) HUVECs infected with adenoviruses coding for Prox1 and COUP-TFII (a), native HDLECs (b) and native HUVECs (c) were subjected to PLA after treating with antibodies for Prox1 and COUP-TFII. Note that specific interaction between Prox1 and COUP-TFII is detected in the nuclei only when Prox1 and COUP-TFII are present. (d) Native HDLECs were subjected to PLA without treating with antibodies for Prox1 and COUP-TFII. Scale bars, 10 μm.

nuclei (Fig. 5Ba), whereas no signals were detected in native HUVECs in the presence of specific antibodies (Fig. 5Bc) or in HDLECs in the absence of specific antibodies (Fig. 5Bd). In the native HDLECs, we could detect definite fluorescence signals restricted to the nuclei in the presence of specific antibodies (Fig. 5Bb), suggesting that endogenous Prox1 and COUP-TFII interact in the nuclei of HDLECs. In order to examine the specificity of the signals, we knocked down COUP-TFII expression by siRNA in HDLECs and carried out PLA (Fig. S3 in Supporting Information). The fluorescence signals seen in the HDLECs transfected with control siRNA were significantly decreased by knocking down COUP-TFII expression. These results allowed us to conclude that endogenous COUP-TFII interacts with Prox1 in the nuclei of HDLECs.

COUP-TFII and Prox1 bind to the cyclin E1 promoter

Petrova *et al.* showed that Prox1 activates cyclin E1 promoter whereas Prox1 DNA binding mutant does not (Petrova *et al.* 2002), suggesting that Prox1 may regulate the transcription of cyclin E1 via direct binding to the cyclin E1 promoter. Because COUP-TFII suppresses Prox1-induced cyclin E1 expression and physically interacts with Prox1, we examined whether Prox1 and COUP-TFII bind to the endogenous cyclin E1 promoter in intact chromatin.

Cross-linked chromatin samples prepared from HUVECs infected with adenoviruses coding for Prox1 and COUP-TFII were subjected to chromatin immunoprecipitation (ChIP) assays (Fig. 6). The cyclin E1 promoter region

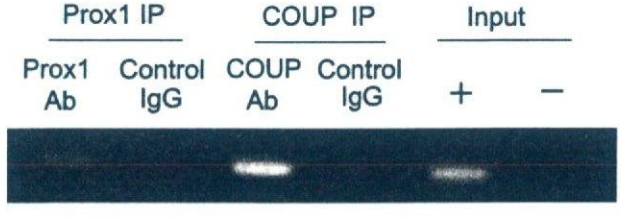

Figure 6 COUP-TFII and Prox1 directly bind to the cyclin E1 promoter. HUVECs infected with adenoviruses coding for Prox1 and COUP-TFII were subjected to ChIP assay. PCR was carried out to detect the cyclin E1 promoter containing putative binding sequences for Prox1 and COUP-TFII. Prox1 Ab and COUP Ab lanes show amplification of target sequences within the immunoprecipitates (IP) using antibodies for Prox1 and COUP-TFII, respectively. Control IgG lanes show PCR amplification of samples precipitated with corresponding control IgG antibodies. Input lanes show amplification of total input DNA (+) or no DNA (–).

containing putative binding consensus sequences for Prox1 and COUP-TFII was pulled down with antibodies for Prox1 and COUP-TFII, suggesting that both Prox1 and COUP-TFII bind to the cyclin E1 promoter.

Discussion

In the present study, we show that COUP-TFII regulates the transcriptional activities of Prox1. In BECs, both gain- and loss-of-function studies showed that COUP-TFII negatively regulates Prox1 to induce the expression of cyclin E1 and VEGFR3. COUP-TFII has been reported to negatively regulate the transcription via binding to the promoter and recruitment of co-repressor complexes containing N-CoR, SMRT and histone deacetylase (HDAC) (Park *et al.* 2003). The present findings that COUP-TFII and Prox1 physically and functionally interact may suggest that positive transcriptional regulation by Prox1 is repressed by the co-repressor complexes that are recruited to the promoter by COUP-TFII.

We also found that endogenous level of COUP-TFII in LECs is required to maintain the expression of VEGFR3, which is not consistent with the results observed in HUVECs. This difference may be caused by the cell type specific contexts of expression of other transcription factors. Additionally, elevation of the level of COUP-TFII expression in LECs also decreased the expression of VEGFR3. We also found that both gain- and loss-of-function of COUP-TFII decreased the expression of LEC markers including integrin $\alpha 9$ and podoplanin and BEC markers including VE-cadherin and VEGFR2 (Fig. S4). These results suggest that a certain range of COUP-TFII expression is required to maintain the expression of a group of LEC and BEC markers. Similar phenomenon is observed in the relationship between the expression of Oct3/4 and maintenance of pluripotency of mouse embryonic stem cells (Niwa *et al.* 2000).

While endothelial markers examined so far appear to require endogenous level of COUP-TFII in HDLECs, we found that not all of endothelial markers are regulated by COUP-TFII in a similar manner. Ablation of COUP-TFII gene in endothelial cells allowed the ectopic expression of ephrin B2 and Neuropilin 1 (NRP1), both of which are arterial endothelial cell markers, in veins (You *et al.* 2005). In accordance with the previous result, COUP-TFII expression decreased ephrin B2 expression while loss-of-COUP-TFII expression increased it in HDLECs (Fig. S4E in Supporting Information). However, to our surprise, both gain- and loss-of-function studies showed that COUP-TFII positively regulates NRP1 expression in HDLECs (Fig. S4F in Supporting Information). Molecular mechanisms of how COUP-TFII differentially regulates the transcription of various target genes between BECs and LECs remain to be investigated in the future.

During embryonic lymphatic development, Prox1 expressing BECs in veins differentiate into LECs and sprout out to form primary lymphatic plexus (Oliver 2004). While we showed that COUP-TFII is expressed in HDLECs at lower level than in HUVECs, it remains to be elucidated how COUP-TFII expression is regulated during lymphatic development in embryos. Loss-of-function studies show that COUP-TFII negatively and positively regulates the VEGFR3 expression in BECs and LECs, respectively. These results may suggest that COUP-TFII maintains the identity of venous endothelial cells by inhibiting Prox1-mediated VEGFR3 expression in BECs, but aids in maintaining VEGFR3 expression in LECs. This differential transcriptional regulation by COUP-TFII may play important roles in segregating lymphatics from veins during formation of primary lymphatic plexus.

As Prox1 plays critical roles in the formation and maintenance of lymphatic vessels (Mishima *et al.* 2007), regulation of transcriptional activities of Prox1 will aid in manupilating lymphangiogenesis in pathological situations. While ligands for COUP-TFII receptor have not yet been identified, better understanding of the regulation of the COUP-TFII may be useful for developing therapeutic strategies to treat lymphedema and tumor lymphangiogenesis in the future.

Experimental procedures

Cell culture and adenovirus production

HUVECs and HDLECs were purchased from Sanko Junyaku and TaKaRa, and cultured in endothelial basal medium (EBM) containing 2% and 5% fetal bovine serum (FBS), respectively, supplemented with endothelial cell growth supplement (TaKaRa). Recombinant adenoviruses coding for mouse Prox1 and mouse COUP-TFII were generated and used as described (Shirakihara *et al.* 2007).

RNA interference and oligonucleotides

siRNAs were introduced into cells using HiperFect reagent (QIAGEN) according to the manufacturer's instructions. The COUP-TFII siRNA (SI00128814) and the negative control siRNA were obtained from QIAGEN.

Immunohistochemistry and Western blot analysis

Immunostaining was carried out with anti-Prox1 (1 : 100 dilution; Abcam), anti-COUP-TFII (1 : 100 dilution; Perseus Proteomics)

and anti-LYVE-1 (1 : 200 dilution; Abcam) antibodies, followed by counterstaining with TOTO3 (Invitrogen–Molecular Probes). Stained specimens were examined using a LSM 510 META confocal microscope (Carl Zeiss). All images were imported into Adobe Photoshop as JPEGs or TIFFs for contrast manipulation and figure assembly. Antibodies to FLAG and α-tubulin for Western blot analysis were obtained from SIGMA. Antibody to human VEGFR3 for Western blot analysis was obtained from Santa Cruz. Western blot analysis was carried out as described (Watabe *et al.* 2003).

Proximity ligation assay (PLA)

The Duolink *in situ* PLA kits were purchased from Olink (http://www.olink.com/). Fixation of the cells, blocking of non-specific binding of antibody, and immunostaining using anti-Prox1 (Abcam) and anti-COUP-TFII (Perseus Proteomics) were carried out as described above. Subsequently, a pair of secondary antibodies conjugated with oligonucleotides (PLA probes) was used according to the manufacturer's protocol to generate fluorescence signals only when the two PLA probes were in close proximity (40 nm). The fluorescence signal from each detected pair of PLA probes was visualized as a distinct individual dot (Söderberg *et al.* 2006, 2008). Nuclei counterstaining and analysis of the images were carried out as described earlier.

Isolation of RNA and quantitative RT-PCR

Total RNAs were extracted from HUVECs and HDLECs using the RNeasy Mini Kit (QIAGEN). First-strand cDNAs were synthesized by SuperScriptIII reverse transcriptase (Invitrogen) using random hexamer primers according to the manufacturer's instruction. Quantitative RT-PCR analyses were carried out using the ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems) and Power SYBR Green PCR master mix (Applied Biosystems). All expression data were normalized to those for β-actin. In the cases when siRNAs and adenoviruses were simultaneously used, expression data are presented as a ratio of the expression level in the samples infected with adenovirus coding for Prox1 against those with non-coding adenovirus. The primer sequences are available online as indicated in Table S1 in Supporting Information.

Chamber migration assay

Migration assay was carried out as described previously (Mishima *et al.* 2007). As a chemoattractant, 100 ng/mL and 300 ng/mL of recombinant VEGF-C (Calbiochem) were used for HDLECs and HUVECs, respectively.

ChIP assays

HUVECs infected with adenoviruses were fixed by adding formaldehyde and harvested. In order to precipitate Prox1 and COUP-TFII, anti-Prox1 antibody (R&D) and anti-COUP-TFII (Perseus Proteomics) were used. PCR of the cyclin E1 promoter containing putative binding sites for Prox1 and COUP-TFII was done using immunoprecipitated chromatin with the following pair of oligonucleotide primers:

5′-ACCAGCCTGAGCAACATAGCA-3′ and 5′-CAGTGAG ACCCCCATTTCTACA-3′.

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References

Cao, R., Björndahl, M.A., Religa, P., Clasper, S., Garvin, S., Galter, D., Meister, B., Ikomi, F., Tritsaris, K., Dissing, S., Ohhashi, T., Jackson, D.G. & Cao, Y. (2004) PDGF-BB induces intratumoral lymphangiogenesis and promotes lymphatic metastasis. *Cancer Cell* **6**, 333–345.

Cui, W., Tomarev, S.I., Piatigorsky, J., Chepelinsky, A.B. & Duncan, M.K. (2004) Mafs, Prox1, and Pax6 can regulate chicken βB1-crystallin gene expression. *J. Biol. Chem.* **279**, 11088–11095.

He, Y., Kozaki, K., Karpanen, T., Koshikawa, K., Yla-Herttuala, S., Takahashi, T. & Alitalo, K. (2002) Suppression of tumor lymphangiogenesis and lymph node metastasis by blocking vascular endothelial growth factor receptor 3 signaling. *J. Natl Cancer Inst.* **94**, 819–825.

Jeltsch, M., Kaipainen, A., Joukov, V., Meng, X., Lakso, M., Rauvala, H., Swartz, M., Fukumura, D., Jain, R.K. & Alitalo, K. (1997) Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science* **276**, 1423–1425.

Kajiya, K., Hirakawa, S., Ma, B., Drinnenberg, I. & Detmar, M. (2005) Hepatocyte growth factor promotes lymphatic vessel formation and function. *EMBO J.* **24**, 2885–2895.

Karkkainen, M.J., Haiko, P., Sainio, K., Partanen, J., Taipale, J., Petrova, T.V., Jeltsch, M., Jackson, D.G., Talikka, M., Rauvala, H., Betsholtz, C. & Alitalo, K. (2004) Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat. Immunol.* **5**, 74–80.

Karpanen, T. & Alitalo, K. (2008) Molecular biology and pathology of lymphangiogenesis. *Annu. Rev. Pathol.* **3**, 367–397.

Mishima, K., Watabe, T., Saito, A., Yoshimatsu, Y., Imaizumi, N., Masui, S., Hirashima, M., Morisada, T., Oike, Y., Araie, M., Niwa, H., Kubo, H., Suda, T. & Miyazono, K. (2007) Prox1 induces lymphatic endothelial differentiation via integrin α9 and other signaling cascades. *Mol. Biol. Cell* **18**, 1421–1429.

Morisada, T., Oike, Y., Yamada,Y., Urano, T., Akao, M., Kubota, Y., Maekawa, H., Kimura, Y., Ohmura, M., Miyamoto, T., Nozawa, S., Koh, G.Y., Alitalo, K. & Suda, T. (2005) Angiopoietin-1 promotes LYVE-1-positive lymphatic vessel formation. *Blood* **105**, 4649–4656.

Niwa, H., Miyazaki, J. & Smith, A.G. (2000) Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat. Genet.* **24**, 372–376.

Oliver, G. (2004) Lymphatic vasculature development. *Nat. Rev. Immunol.* **4**, 35–45.

Park, J.I., Tsai, S.Y. & Tsai, M.J. (2003) Molecular mechanism of chicken ovalbumin upstream promoter-transcription factor (COUP-TF) actions. *Keio J. Med.* **52**, 174–181.

Petrova, T.V., Mäkinen, T., Mäkelä, T.P., Saarela, J., Virtanen, I., Ferrell, R.E., Finegold, D.N., Kerjaschki, D., Ylä-Herttuala, S. & Alitalo, K. (2002) Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. *EMBO J.* **21**, 4593–4599.

Qin, J., Gao, D.M., Jiang, Q.F., Zhou, Q., Kong, Y.Y., Wang, Y. & Xie, Y.H. (2004) Prospero-related homeobox (Prox1) is a corepressor of human liver receptor homolog-1 and suppresses the transcription of the cholesterol 7-α-hydroxylase gene. *Mol. Endocrinol.* **18**, 2424–2439.

Shin, J.W., Min, M., Larrieu-Lahargue, F., Canron, X., Kunstfeld, R., Nguyen, L., Henderson, J.E., Bikfalvi, A., Detmar, M. & Hong, Y.K. (2006) Prox1 promotes lineage-specific expression of fibroblast growth factor (FGF) receptor-3 in lymphatic endothelium: a role for FGF signaling in lymphangiogenesis. *Mol. Biol. Cell* **17**, 576–584.

Shirakihara, T., Saitoh, M. & Miyazono, K. (2007) Differential regulation of epithelial and mesenchymal markers by δEF1 proteins in epithelial mesenchymal transition induced by TGF-β. *Mol. Biol. Cell* **18**, 3533–3544.

Söderberg, O., Gullberg, M., Jarvius, M., Ridderstråle, K., Leuchowius, K.J., Jarvius, J., Wester, K., Hydbring, P., Bahram, F., Larsson, L.G. & Landegren, U. (2006) Direct observation of individual endogenous protein complexes *in situ* by proximity ligation. *Nat. Methods* **3**, 995–1000.

Söderberg, O., Leuchowius, K.J., Gullberg, M., Jarvius, M., Weibrecht, I., Larsson, L.G. & Landegren, U. (2008) Characterizing proteins and their interactions in cells and tissues using the *in situ* proximity ligation assay. *Methods* **45**, 227–232.

Srinivasan, R.S., Dillard, M.E., Lagutin, O.V., Lin, F.J., Tsai, S., Tsai, M.J., Samokhvalov, I.M. & Oliver, G. (2007) Lineage tracing demonstrates the venous origin of the mammalian lymphatic vasculature. *Genes Dev.* **21**, 2422–2432.

Steffensen, K.R., Holter, E., Båvner, A., Nilsson, M., Pelto-Huikko, M., Tomarev, S. & Treuter, E. (2004) Functional conservation of interactions between a homeodomain cofactor and a mammalian FTZ-F1 homologue. *EMBO Rep.* **5**, 613–619.

Veikkola, T., Jussila, L., Makinen, T., Karpanen, T., Jeltsch, M., Petrova, T.V., Kubo, H., Thurston, G., McDonald, D.M., Achen, M.G., Stacker, S.A. & Alitalo, K. (2001) Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J.* **20**, 1223–1231.

Vlahakis, N.E., Young, B.A., Atakilit, A. & Sheppard, D. (2005) The lymphangiogenic vascular endothelial growth factors VEGF-C and -D are ligands for the integrin α9β1. *J. Biol. Chem.* **280**, 4544–4552.

Watabe, T., Nishihara, A., Mishima, K., Yamashita, J., Shimizu, K., Miyazawa, K., Nishikawa, S. & Miyazono, K. (2003) TGF-β receptor kinase inhibitor enhances growth and integrity of embryonic stem cell-derived endothelial cells. *J. Cell Biol.* **163**, 1303–1311.

Wigle, J.T., Harvey, N., Detmar, M., Lagutina, I., Grosveld, G., Gunn, M.D., Jackson, D.G. & Oliver, G. (2002) An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *EMBO J.* **21**, 1505–1513.

Wigle, J.T. & Oliver, G. (1999) Prox1 function is required for the development of the murine lymphatic system. *Cell* **98**, 769–778.

You, L.R., Lin, F.J., Lee, C.T., DeMayo, F.J., Tsai, M.J. & Tsai, S.Y. (2005) Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. *Nature* **435**, 98–104.

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Supporting Information/Supplementary material

The following Supporting Information can be found in the online version of the article:

Figure S1 Efficacy of knockdown of COUP-TFII expression in HUVECs.

Figure S2 COUP-TFII suppresses Prox1-induced cyclin E2 expression in HUVECs.

Figure S3 Interaction of endogenous Prox1 and COUP-TFII in HDLECs.

Figure S4 Endogenous level of COUP-TFII expression plays important roles in the maintenance of characteristics of HDLECs.

Table S1 Primers used for RT-PCR

Additional Supporting Information may be found in the online version of the article.

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Snail is required for TGFβ-induced endothelial-mesenchymal transition of embryonic stem cell-derived endothelial cells

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Summary

Epithelial-mesenchymal transition (EMT) plays important roles in various physiological and pathological processes, and is regulated by signaling pathways mediated by cytokines, including transforming growth factor β (TGFβ). Embryonic endothelial cells also undergo differentiation into mesenchymal cells during heart valve formation and aortic maturation. However, the molecular mechanisms that regulate such endothelial-mesenchymal transition (EndMT) remain to be elucidated. Here we show that TGFβ plays important roles during mural differentiation of mouse embryonic stem cell-derived endothelial cells (MESECs). TGFβ2 induced the differentiation of MESECs into mural cells, with a decrease in the expression of the endothelial marker claudin 5, and an increase in expression of the mural markers smooth muscle α-actin, SM22α and calponin, whereas a TGFβ type I receptor kinase inhibitor inhibited EndMT. Among the transcription factors involved in EMT, Snail was induced by TGFβ2 in MESECs. Tetracycline-regulated expression of Snail induced the differentiation of MESECs into mural cells, whereas knockdown of *Snail* expression abrogated TGFβ2-induced mural differentiation of MESECs. These results indicate that Snail mediates the actions of endogenous TGFβ signals that induce EndMT.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/121/20/3317/DC1

Key words: TGFβ2, TβR-I inhibitor, Snail, EMT, EndMT, Embryonic stem cell, Claudin 5, Smooth muscle α-actin

Introduction

Blood vessels are lined by endothelial cells and, with the exception of capillaries, are surrounded by mural cells (pericytes or smooth muscle cells). Embryonic endothelial cells arise from mesodermal cells that express vascular endothelial growth factor receptor 2 (VEGFR2; also known as KDR) (Carmeliet 2005; Coultas et al., 2005). Previous studies have described embryonic mural cells as originating from neural crest and locally differentiating mesenchymal cells (Hirschi and Majesky, 2004). Although embryonic stem cell (ESC)-derived vascular progenitor cells have been shown to differentiate into both endothelial and mural cells (Yamashita et al., 2000; Marchetti et al., 2002; Ema et al., 2003), there is no direct evidence to show that mural cells differentiate from common vascular progenitor cells in vivo.

Differentiated endothelial cells have been shown to differentiate into mesenchymal cells in vivo. During heart development, cardiogenic mesodermal cells give rise to two types of heart cells: myocardial and endocardial cells. Endocardial cells acquire endothelial markers, such as VE-cadherin (cadherin 5) and platelet-endothelial cell adhesion molecule 1 (PECAM1). A population of endocardial/endothelial cells in the atrioventricular (AV) canal gives rise to the mesenchymal heart cushion cells, which form the mesenchymal region of cardiac septa and valves (Markwald et al., 1977; Potts et al., 1991). Furthermore, during maturation of dorsal aorta in quail, endothelial cells experimentally labeled with a wheat germ agglutinin-colloidal gold marker were shown to differentiate into subendothelial mesenchymal cells that were positive for both endothelial and mural markers in the aortic wall (DeRuiter et al., 1997; Arcinegas et al., 2000).

During these processes, endothelial cells undergo a process similar to epithelial-mesenchymal transition (EMT). EMT converts polarized epithelial cells into motile mesenchymal cells (Lee et al., 2006). EMT plays important roles in gastrulation and cancer cell invasion (Huber et al., 2005). During EMT, the epithelial markers E-cadherin (cadherin 1) and zona occludens 1 (ZO1; also known as TJP1) are downregulated, whereas the mesenchymal markers smooth muscle α-actin (SMA) and vimentin are upregulated. During endothelial-mesenchymal transition (EndMT) in heart development, expression of VE-cadherin is downregulated, whereas that of SMA is upregulated. EndMT in heart valve formation is regulated by signaling pathways mediated by multiple cytokines, including Wnt, Notch and transforming growth factor β (TGFβ).

Members of the TGFβ family bind to two different types of serine/threonine kinase receptors. Upon ligand binding, the constitutively active type II receptor kinase phosphorylates the type I receptor, which, in turn, activates the downstream signal transduction cascades, including Smad pathways. Activins and TGFβs signal through the type I receptors known as activin receptor type B (ACVR1B, hereafter referred to as ALK4) and transforming growth factor beta receptor 1 (TGFβR1; hereafter referred to as ALK5), respectively. The activated type I receptors phosphorylate receptor-regulated Smad proteins (R-Smads). Smad2 and 3 transduce signals for TGFβs and activins, whereas Smad1, 5 and 8 (also known as Smad9) are specific for signaling of bone

morphogenetic proteins (BMPs) (Feng and Derynck, 2005). As an exception, ALK1 (ACVRL1), which is preferentially expressed in endothelial cells, binds TGFβ and activates Smad1/5 pathways (Oh et al., 2000). Recently, BMP9 and BMP10 were reported to bind to ALK1 (David et al., 2007; Scharpfenecker et al., 2007). Once activated, R-Smads form a complex with the common mediator Smad4 (co-Smad) and translocate to the nucleus, where Smad complexes regulate transcription of target genes through their interaction with various transcription factors. In addition, TGFβ has been shown to activate a diversity of non-Smad parallel downstream pathways, including extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK) and p38 MAP kinase (Derynck and Zhang, 2003).

Knockout mice deficient in various TGFβ family signaling components exhibit defects in cardiovascular tissues, implicating a role for TGFβ family proteins in cardiovascular development (Goumans and Mummery, 2000; Mercado-Pimentel and Runyan, 2007). In particular, TGFβ2-deficient mice have multiple defects in AV cushion formation, suggesting a role in EndMT of endocardiac tissues (Sanford et al., 1997; Bartram et al., 2001). Furthermore, various in vitro studies have shown that TGFβs induce the differentiation of vascular endothelial cells into mural cells (Arciniegas et al., 1992; Frid et al., 2002; Ishisaki et al., 2003). However, the molecular mechanisms that govern TGFβ-induced EndMT remain largely unknown.

Several transcription factors, including Snail (SNAI1), Slug (SNAI2), δEF1 (ZEB1), SIP1 and Twist, have been implicated in EMT (Peinado et al., 2007). Snail, a zinc-finger-containing transcription factor, represses E-cadherin expression and induces EMT when overexpressed in epithelial cells (Cano et al., 2000; Batlle et al., 2000). Knockout mice deficient for *Snail* die at gastrulation because they fail to undergo a complete EMT process, forming an abnormal mesodermal layer that maintains E-cadherin expression (Carver et al., 2001). Although TGFβ signals have been shown to induce the expression of Snail, SIP1 and δEF1 during EMT of mammary epithelial cells (Peinado et al., 2003; Shirakihara et al., 2007), the causal relationship between TGFβ-induced Snail expression and EMT has not yet been fully elucidated.

In order to elucidate the roles of TGFβ signaling in the differentiation of embryonic endothelial cells into mural cells, we used endothelial cells derived from mouse ESCs. TGFβ2 decreased the expression of an endothelial marker, claudin 5, and increased that of mural markers, SMA, SM22α (transgelin) and calponin. We also found that Snail is necessary for the TGFβ2-induced mural differentiation of endothelial cells. These results reveal that Snail plays important roles in the TGFβ-induced EndMT.

Results

TGFβ and activin induce differentiation of ESC-derived endothelial cells into SMA-expressing cells

When VEGFR2-expressing vascular progenitor cells derived from mouse ESCs were cultured for 3 days with VEGF (VEGFA), we obtained cells positive for mural cell marker SMA, which surround endothelial cells positive for PECAM1 and CD34 (Yamashita et al., 2000). These mixed vascular cell populations were sorted using anti-CD34 antibodies in order to purify endothelial cells. The proportion of PECAM1$^+$ cells in the sorted population was nearly 100% (data not shown). We used these mouse ESC-derived endothelial cells (MESECs) in the present study (supplementary material Fig. S1).

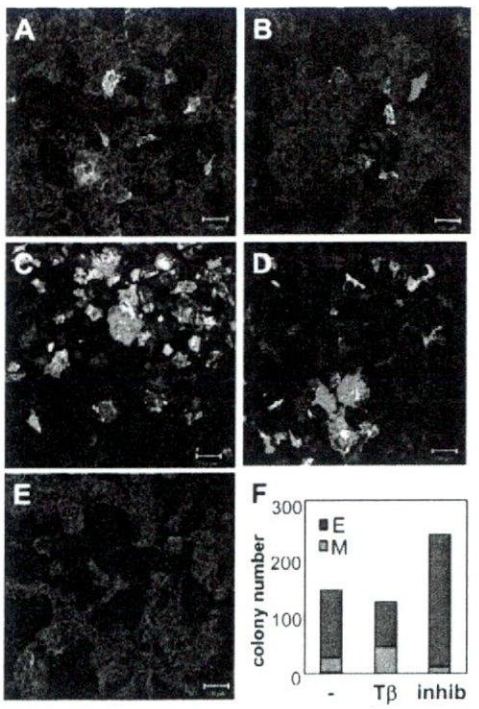

Fig. 1. Effects of TGFβ family members on mouse ESC-derived endothelial cells (MESECs). (A-E) MESECs were obtained by CD34-sorting of vascular (endothelial and mural) cells derived from mouse ESCs, and cultured in the presence of 10% fetal calf serum (FCS) and VEGF (A), followed by immunofluorescence staining for PECAM1 (red) and SMA (green). MESECs were also treated with BMP4 (B), TGFβ2 (C), activin (D) and TβR-I inhibitor (E). Scale bars: 100 μm. (F) Quantitative analysis of the effects of TGFβ signals on colony formation from single MESECs. MESECs were cultured at low density with 10% FCS and VEGF in the absence (–) or presence of TGFβ2 (Tβ) or TβR-I inhibitor (inhib) for 4 days, and then stained for PECAM1 and SMA. The number of colonies per well was counted to assess the effect of TGFβ signals on colony formation of MESECs. Two colony types were observed: those consisting of pure endothelial cells (E) and those also containing mural cells (M). Experiments were repeated at least three times with essentially the same results.

After MESECs were cultured for 4 days in the presence of VEGF, the proportion of endothelial cells remained higher than 95% (Fig. 1A). Previous reports have shown that cultured endothelial cells differentiate into mesenchymal cells expressing SMA under long-term stimulation by TGFβ (Paranya et al., 2001; Frid et al., 2002). In order to study the effects of TGFβ on MESECs, we used TGFβ2, which seems to be the physiologically most relevant TGFβ isoform for EndMT during heart cushion development (Camenisch et al., 2002), as well as activin and BMP4, other members of the TGFβ family. Whereas BMP4 did not exhibit significant effects (Fig. 1B), TGFβ2 and activin led to a decrease in the number of PECAM1$^+$ sheets of endothelial cells and to an increase in SMA$^+$ cells (Fig. 1C,D). We also compared the effects of different isoforms of TGFβ on MESECs and found that TGFβ1 and 3 induced the EndMT in a similar manner to TGFβ2 (supplementary material Fig. S2).

Furthermore, inhibition of endogenously activated TGFβ/activin signals in MESECs (Watabe et al., 2003) by a small-molecule that inhibits kinases of receptors for TGFβ, actin and nodal (TβR-I inhibitor, also known as LY364947) (Sawyer et al., 2003), led to a decrease in SMA$^+$ cells (Fig. 1E). These results

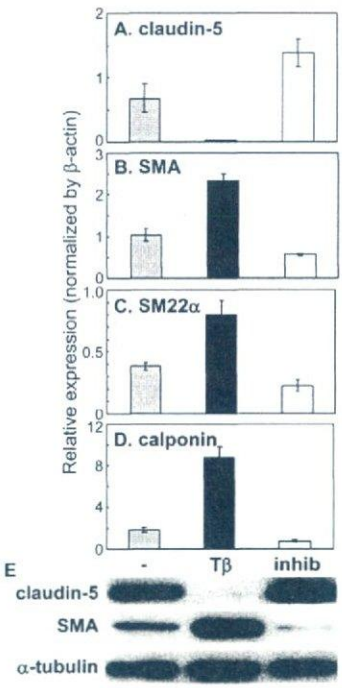

Fig. 2. Effect of TGFβ signals on expression of endothelial and mural markers in MESECs. (A-D) Levels of mRNA expression for claudin 5 (A), SMA (B), SM22α (C) and calponin (D) in MESECs cultured in the absence (–) or presence of TGFβ2 (Tβ) or TβR-I inhibitor (inhib) as analyzed by quantitative real-time RT-PCR. Error bars indicate s.d. (E) Protein levels of claudin 5 (top), SMA (middle) and α-tubulin (bottom) were examined by immunoblotting of total lysates of the MESECs described in A-D.

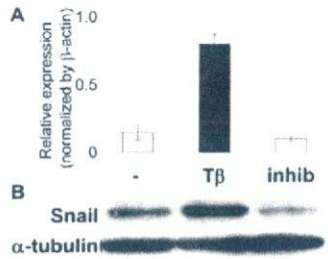

Fig. 3. Effect of TGFβ signals on Snail expression in MESECs. (A) Levels of expression of Snail in MESECs cultured in the absence (–) or presence of TGFβ2 (Tβ) or TβR-I inhibitor (inhib) as analyzed by quantitative real-time RT-PCR. Error bars indicate s.d. (B) Protein levels of Snail (top) and α-tubulin (bottom) were examined by immunoblotting of total lysates of the HUVECs cultured in the absence or presence of TGFβ2 or TβR-I inhibitor.

suggest that activation of ALK5-mediated signaling pathways induces the differentiation of MESECs into SMA-expressing cells.

Quantitative analysis of the effects of TGFβ signals on the differentiation of MESECs into SMA-expressing cells

In order to further dissect the roles of TGFβ in the differentiation of MESECs into SMA^+ cells, we performed quantitative analysis using a limiting-dilution assay. When MESECs were plated at a low density, single MESECs formed individual colonies, and the total number of colonies varied depending on the culture conditions. TGFβ2 decreased the number of colonies slightly, whereas the TβR-I inhibitor strongly increased it (Fig. 1F).

We next evaluated the phenotypes of the colonies by immunohistochemical analysis. Culturing MESECs with VEGF induced two types of colonies emerging from single MESECs: $PECAM1^+$ pure endothelial cells (E), and mixtures of endothelial and SMA^+ mural cells (M) (Fig. 1F). Whereas the frequency of mural colonies was 19% when cultured in the presence of VEGF, addition of TGFβ2 or TβR-I inhibitor reproducibly increased (to 37%) or decreased (to 5%) this frequency, respectively, further suggesting that TGFβ signals induce the differentiation of MESECs into SMA^+ cells.

TGFβ regulates the expression of various endothelial and mural cell markers

Vascular endothelial cells express various markers, including PECAM1, VE-cadherin and claudin 5, whereas mural cells express markers such as SMA, SM22α and calponin. During the EndMT in heart cushion development, expression of VE-cadherin is downregulated with concomitant upregulation of SMA expression. We studied the effects of TGFβ signals on the expression of various markers for endothelial and mural cells in MESECs using quantitative RT-PCR and immunoblot analyses. Whereas the expression of VE-cadherin was unaffected by TGFβ2 (data not shown), TGFβ2 treatment resulted in a decrease in claudin 5 expression (Fig. 2A) with a concomitant increase in SMA expression at both the mRNA (Fig. 2B) and protein (Fig. 2E) levels. By contrast, addition of TβR-I inhibitor increased the expression of claudin 5 and decreased SMA expression (Fig. 2A,B,E).

We also studied the expression of other mural cell markers. As shown in Fig. 2C,D, SM22α and calponin were both upregulated by TGFβ2 and downregulated by TβR-I inhibitor. These results suggest that TGFβ2 induces the EndMT of MESECs.

TGFβ induces the expression of Snail during mural cell differentiation of MESECs

Recent studies have revealed that several transcription factors are involved in EMT (Peinado et al., 2007). In order to elucidate the molecular mechanisms that govern TGFβ-induced EndMT of MESECs, we studied the expression of various EMT-related transcription factors after treatment of MESECs with TGFβ2 or TβR-I inhibitor. As shown in Fig. 3A, expression of Snail was significantly increased by TGFβ2 treatment, whereas it was slightly decreased by the addition of TβR-I inhibitor. By contrast, expression of Slug, SIP1, δEF1 or Twist, was unaffected by TGFβ2 treatment in MESECs (data not shown).

We further studied the effect of TGFβ signals on the expression of Snail protein in human umbilical vein endothelial cells (HUVECs). In accordance with the results in MESECs, expression of Snail was induced by TGFβ2 and decreased by TβR-I inhibitor (Fig. 3B). These results suggest Snail as a possible regulator of EndMT in response to TGFβ2 treatment.

Snail expression in MESECs induces EndMT

Since Snail expression was induced by TGFβ, we examined the effects of Snail expression on MESECs. Because we wished to induce the expression of Snail in differentiated endothelial cells instead of undifferentiated ESCs, we established ESC lines carrying a tetracycline (Tc)-regulatable Snail transgene (Tc-Snail), or no transgene (Tc-Empty) (Masui et al., 2005; Mishima et al., 2007).

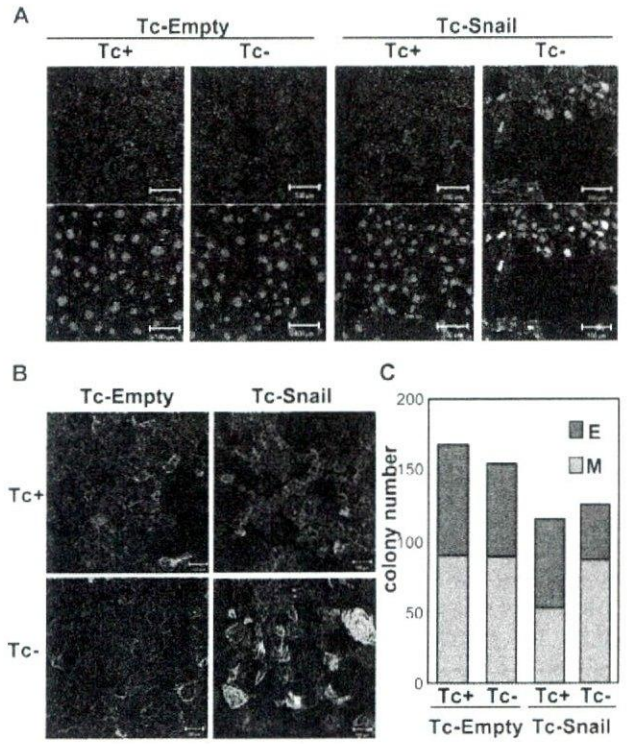

Fig. 4. Effect of tetracycline (Tc)-regulated Snail expression on MESECs. (A) MESECs were sorted from the vascular cells derived from ESCs carrying a Tc-regulated transgene encoding FLAG-epitope-tagged mouse Snail (Tc-Snail) or control transgene (Tc-Empty), and cultured in the absence (–) or presence (+) of Tc. Expression of FLAG-Snail (top and bottom rows, green) was examined, in addition to nuclear staining (bottom row, red). (B) MESECs in A were subject to immunofluorescence staining for PECAM1 (red) and SMA (green). (C) Quantitative analysis of the effects of Snail on colony formation from single MESECs, performed as described in Fig. 1F. Briefly, MESECs derived from Tc-Empty or Tc-Snail ESCs were cultured at low density with 10% FCS in the absence (–) or presence (+) of Tc for 4 days, followed by staining of colonies for PECAM1 and SMA. E, pure endothelial colony; M, mural-containing colony. Scale bars: 100 μm.

EMT has been reported to play important roles during the early stages of development, such as gastrulation. Recently, upregulation of Snail expression was detected during an early differentiation process of human ESCs, which was identified as EMT (Ullmann et al., 2007). Removal of Tc from the culture of undifferentiated Tc-Snail cells, but not that of Tc-Empty cells, induced the expression of the Snail transgene, which resulted in dramatic changes in their morphology from epithelial to mesenchymal in appearance (supplementary material Fig. S3A). We further found that E-cadherin expression was downregulated by Snail expression (supplementary material Fig. S3B). These results suggest that Snail enhances the EMT that takes place during early differentiation process of ESCs.

In order to examine the effects of Snail on vascular development, we differentiated the Tc-Empty and Tc-Snail ESCs into vascular cells in the presence of Tc, so that no transgene expression was induced until the endothelial cells were fully differentiated. MESECs were sorted using anti-CD34 antibodies, and were cultured in the presence or absence of Tc (supplementary material Fig. S1). As shown in Fig. 4A, Snail transgene expression was induced in the vascular cells derived from Tc-Snail ESCs only in the absence of Tc. The majority of control MESECs maintained their endothelial characteristics, expressing PECAM1 when Snail was not expressed (Fig. 4B). However, when Snail was expressed, many of the endothelial cells exhibited mural characteristics, including expression of SMA (Fig. 4B), as observed when MESECs were treated with TGFβ2 (Fig. 1).

To further dissect the roles of Snail in the EndMT of MESECs, we performed quantitative colony-formation assays. The frequency of colonies containing SMA^+ cells (M) was unaffected by the removal of Tc in the MESECs derived from Tc-Empty ESCs (Tc+, 53%; Tc–, 57%) (Fig. 4C). By contrast, Snail expression induced by the removal of Tc in the Tc-Snail ESC-derived MESECs significantly increased the frequency of SMA^+ colony formation (Tc+, 45%; Tc–, 69%). This suggests that Snail induces the differentiation of MESECs into SMA^+ cells.

We examined whether induction of EndMT by Snail transgene expression requires activation of TGFβ type I receptors. Addition of TβR-I inhibitor to the MESECs derived from Tc-Snail ESCs in the presence of Tc abrogated the EndMT induced by endogenous TGFβ signals (supplementary material Fig. S4A). Expression of the Snail transgene by removal of Tc induced EndMT, both in the absence and presence of TβR-I inhibitor. These results were confirmed by a quantitative colony-formation assay (supplementary material Fig. S4B) and suggest that Snail induces the EndMT of MESECs as a downstream target of TGFβ type I receptor-mediated signals.

Snail downregulates endothelial marker expression and upregulates mural marker expression

We further examined the effects of Snail on the expression of various endothelial and mural cell markers in MESECs. As shown in Fig. 5A,B, the amount of transcript for claudin 5 and for SMA was down- and upregulated, respectively, by Snail expression in the MESECs derived from Tc-Snail ESCs. Analogous changes in protein levels were confirmed by immunoblot analysis (Fig. 5E).

The expression of other mural cell markers, SM22α and calponin, was also upregulated by Snail expression in MESECs (Fig. 5C,D). These changes in expression of the various markers induced by Snail are reminiscent of those induced by TGFβ2 treatment, suggesting that Snail is involved in the TGFβ2-induced EndMT of MESECs.

Snail is required for the TGFβ2-induced EndMT of MESECs

In order to determine whether Snail is required for the TGFβ2-mediated EndMT, we used siRNA directed against *Snail* to reduce the expression of endogenous protein. Snail siRNA was transfected into MESECs, followed by stimulation of the cells with TGFβ2 (supplementary material Fig. S1). As shown in Fig. 6A, Snail siRNA successfully knocked down the expression of endogenous *Snail* mRNA while TGFβ2 still induced *Snail* expression to the endogenous level. In cells transfected with control siRNA, TGFβ2 induced the differentiation of MESECs into SMA^+ cells, whereas TGFβ2 failed to do so in the cells transfected with Snail siRNA (Fig. 6B). These results were confirmed by quantitative colony-formation assays (Fig. 6C).

Furthermore, the TGFβ2-mediated upregulation of expression of various mural cell markers (SMA, SM22α and calponin) was repressed by Snail siRNA, whereas downregulation of claudin 5 expression by TGFβ2 was partially inhibited by Snail siRNA (Fig. 7A-E). These results reveal that Snail is necessary for the EndMT induced by TGFβ2.

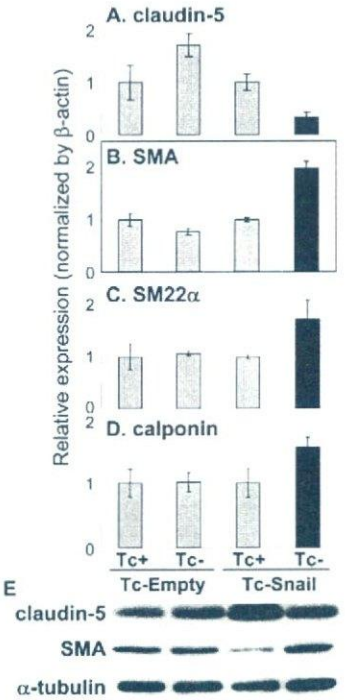

Fig. 5. Effect of Snail on expression of endothelial and mural markers in MESECs. (A-D) Levels of expression of claudin 5 (A), SMA (B), SM22α (C) and calponin (D) in MESECs derived from Tc-Empty or Tc-Snail ESCs cultured in the absence (–) or presence (+) of Tc were analyzed by quantitative real-time RT-PCR. Error bars indicate s.d. (E) Protein levels of claudin 5 (top), SMA (middle) and α-tubulin (bottom) were examined by immunoblotting of total lysates of the MESECs described in A-D.

Smad4-independent pathways are partly involved in the TGFβ2-induced EndMT of MESECs

Upon ligand binding, TGFβ receptor complexes activate both Smad and non-Smad signaling pathways. In order to examine whether these non-Smad pathways are involved in the TGFβ-induced EndMT, we knocked down the expression of *Smad4*, the only co-Smad that is necessary for both the Smad2/3 and Smad1/5/8 pathways (Fig. 8A). In the MESECs in which *Smad4* expression was knocked down, TGFβ2 failed to induce the expression of PAI1 (SERPINE1) (Fig. 8B), a target of the Smad2/3 pathway, but partially induced Snail expression (Fig. 8C), suggesting that Snail is partially induced by Smad4-independent pathways. In accordance with the results of Snail expression, knockdown of *Smad4* expression failed to fully abrogate the TGFβ2-mediated EndMT (Fig. 8D), the suppression of claudin 5 (Fig. 8E) or the induction of SMA expression (Fig. 8F). These results suggest that TGFβ activates Smad4-dependent and -independent pathways, both of which play important roles in the induction of Snail expression that leads to EndMT.

We also examined whether the induction of EndMT by Snail transgene expression requires Smad4. The expression of the Snail transgene by removal of Tc from the culture of the MESECs derived from Tc-Snail ESCs was able to induce EndMT, both in the absence and presence of Smad4 expression (supplementary material Fig. S5A). These results were confirmed by quantitative RT-PCR (supplementary material Fig. S5B,C), and suggest that Snail induces the EndMT of MESECs as a downstream target of Smad4-mediated signals.

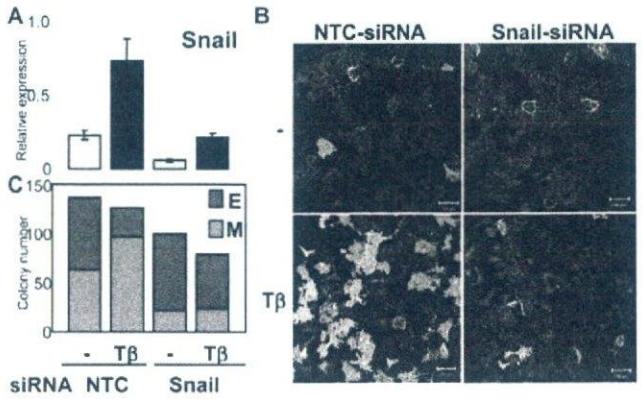

Fig. 6. Effect of *Snail* knockdown on MESECs. MESECs were sorted from the vascular cells derived from ESCs, transfected with Snail siRNA or with scrambled sequence as a negative control (NTC), and cultured in the absence (–) or presence of TGFβ2 (Tβ). (A) The levels of endogenous expression of Snail in the MESECs were analyzed by quantitative real-time RT-PCR. Error bars indicate s.d. Black and gray bars, represent +TGFβ2 and –TGFβ2, respectively. (B) The MESECs were subjected to immunofluorescence staining for PECAM1 (red) and SMA (green). (C) Quantitative analysis of the effects of Snail on colony formation from single MESECs, performed as described in Fig. 1F. Briefly, MESECs transfected with Snail siRNA or scrambled sequence were cultured at low density with 10% FCS in the absence (–) or presence of TGFβ2 (Tβ) for 4 days, followed by staining of colonies for PECAM1 and SMA. E, pure endothelial colony; M, mural-containing colony. Scale bars: 100 μm.

Discussion

In the present study, we showed that TGFβ2 induces the differentiation of endothelial cells into mural cells, with an increase in expression of the mural markers, SMA, SM22α and calponin. Previous reports have shown that TGFβ induces various mural markers during the differentiation of neural crest stem cells into smooth muscle cells (Shah et al., 1996), and that TGFβ-induced δEF1 is involved in this process (Nishimura et al., 2006). Although TGFβ has been shown to induce the expression of Snail during EMT of kidney epithelial cells (Peinado et al., 2003), functional roles of Snail during TGFβ-induced EMT were not fully elucidated. The present findings directly show, for the first time, that Snail mediates TGFβ-induced upregulation of multiple mural markers and the downregulation of claudin 5 in endothelial cells.

We also found that loss of Smad4 expression decreases, but does not completely abolish, TGFβ-induced Snail expression and EndMT (Fig. 8). We previously showed that Snail expression is upregulated within 30 minutes of addition of TGFβ to NMuMG mammary epithelial cells, in which TGFβ induces EMT (Shirakihara et al., 2007), suggesting that Snail is a direct target of TGFβ signals. The molecular mechanisms by which Smad4-dependent and -independent signals activate the Snail promoter in endothelial cells remain to be studied in the future.

Although Snail has been shown to play important roles in EMT, the molecular mechanisms by which Snail regulates the transcription of EMT-related targets have not been elucidated. In order to examine whether Snail binds to the endogenous SMA promoters in intact chromatin, we have subjected cross-linked chromatin samples prepared from Tc-Snail ESC-derived endothelial cells to chromatin immunoprecipitation (ChIP) assays. Nishimura and colleagues previously identified a TGFβ-responsive SMA promoter region containing Smad3-binding sequences and an E-box to which

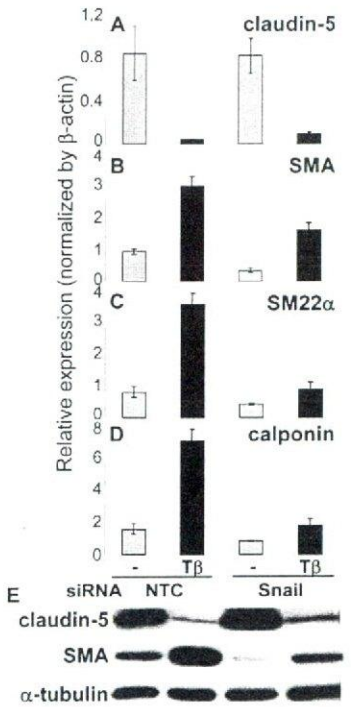

Fig. 7. Effect of Snail on expression of endothelial and mural markers in MESECs. (A-D) Levels of expression of claudin 5 (A), SMA (B), SM22α (C) and calponin (D) in MESECs transfected with Snail siRNA or scrambled sequence as a negative control (NTC), and cultured in the absence (–) or presence of TGFβ2 (Tβ) were analyzed by quantitative real-time RT-PCR. Error bars indicate s.d. (E) Protein levels of claudin 5 (top), SMA (middle) and α-tubulin (bottom) were examined by immunoblotting of total lysates of the MESECs described in A-D.

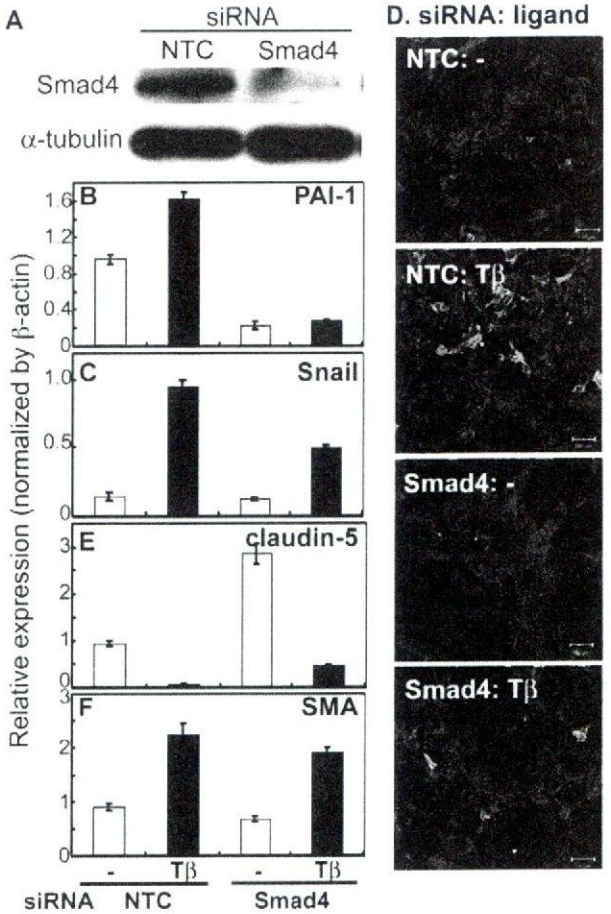

Fig. 8. Effect of *Smad4* knockdown on TGFβ-induced EndMT of MESECs. MESECs were sorted from the vascular cells derived from ESCs, transfected with Smad4 siRNA or with scrambled sequence as a negative control (NTC), and cultured in the absence (–) or presence of TGFβ2 (Tβ). (A) Levels of endogenous expression of Smad4 in the MESECs were analyzed by immunoblotting. (B,C,E,F) Levels of expression of PAI1 (B), Snail (C), claudin 5 (E) and SMA (F) in the MESECs were analyzed by quantitative real-time RT-PCR. Error bars indicate s.d. (D) The MESECs were subject to immunofluorescence staining for PECAM1 (red) and SMA (green).

Snail proteins might bind (Nishimura et al., 2006). We were also able to pull down the TGFβ-responsive element with antibodies against Smad3 in the Tc-Snail ESC-derived endothelial cells treated with TGFβ (supplementary material Fig. S6A), but not with antibodies against FLAG-Snail (supplementary material Fig. S6B). These results suggest that Snail does not bind to the TGFβ-responsive element to induce SMA expression in MESECs.

During EMT, a decrease in the expression of multiple tight-junction molecules, such as ZO1 and claudins, is accompanied by an increase in the expression of mesenchymal markers. We observed a decrease in the expression of claudin 5, an endothelium-specific tight-junction molecule, induced by TGFβ in MESECs. We previously reported that expression of claudin 5 is downregulated by TGFβ during endothelial differentiation from ESC-derived vascular progenitor cells (Watabe et al., 2003). Since claudin 1 expression is also repressed by Snail and Slug during EMT of kidney epithelial cells (Martinez-Estrada et al., 2006), downregulation of claudin family members might be a crucial event during EMT and EndMT.

During EMT and EndMT, expression of E-cadherin and VE-cadherin is, respectively, also decreased. However, VE-cadherin expression was not altered by Snail in MESECs, whereas E-cadherin expression was suppressed by Snail in undifferentiated ESCs. This might suggest that repression of VE-cadherin requires other transcription factors. We recently showed that TGFβ-induced δEF1 and SIP1, but not Snail, are involved in the downregulation of E-cadherin expression in mammary epithelial cells (Shirakihara et al., 2007). However, expression of the other EMT-related transcription factors was unaffected by TGFβ in MESECs, suggesting that other EMT-related signaling pathways are involved in the repression of VE-cadherin expression. In the embryonic heart, Notch functions to promote the TGFβ-induced EMT that results in formation of the cardiac valvular primordia (Timmerman et al., 2004). Liebner and colleagues showed that TGFβ induction of EndMT during heart cushion development is strongly inhibited in mice deficient for β-catenin, suggesting that an interaction between TGFβ and Wnt signaling pathways plays important roles in this process (Liebner et al., 2004). The roles of Notch and Wnt signals in the EndMT of MESECs remain to be elucidated in the future.

The expression of Twist, another EMT-related transcription factor, has been reported to be regulated by BMP2 (Ma et al., 2005), a member of the TGFβ family that has been implicated in cardiac cushion EndMT. In the embryos that lack BMP2 or BMP type IA receptor in AV myocardium or endocardium, respectively, cardiac

cushion formation is perturbed, with loss of expression of various transcription factors, including Twist (Ma et al., 2005). However, the present study shows that BMP4 fails to induce EndMT of MESECs. In accordance with this, Snail expression in the endocardium was unaffected by the loss of BMP2 or BMP type IA receptor (Ma et al., 2005). A recent report showed that BMP7 inhibits the TGFβ-induced EndMT of cardiac endothelial cells (Zeisberg et al., 2007a). We also found that BMP7 partially inhibits the TGFβ-mediated SMA expression in MESECs (supplementary material Fig. S7). These results suggest that certain types of BMPs play roles in the EndMT in a manner independent of TGFβ.

Recently, EndMT was implicated in two pathological situations. During cardiac fibrosis, accumulated fibroblasts cause the deposition of extracellular matrix, which can cause heart failure. Furthermore, activated fibroblasts can induce the progression of cancers. Zeisberg and colleagues reported that the TGFβ-induced EndMT plays important roles in the formation of fibroblasts from endothelial cells during cardiac fibrosis (Zeisberg et al., 2007a) and cancer progression (Zeisberg et al., 2007b). Since fibroblasts are key to both situations, EndMT is expected to be a target in the therapy of cardiac dysfunction and cancer. Therefore, the present findings might lead to a greater understanding of not only normal cardiovascular development, but also of such pathological situations, and eventually to the development of strategies to manipulate these signals for therapeutic benefit.

Materials and Methods

Cells and cell culture

The maintenance, differentiation, culture and cell sorting of mouse CCE and MGZ5TcH2 ESCs (gifts from Drs M. J. Evans and H. Niwa, respectively) were as described (Yamashita et al., 2000). Differentiated ESC-derived endothelial cells were sorted using PE-conjugated anti-CD34 antibodies (Pharmingen) and a MACS separation system (Miltenyi Biotec). Establishment of Tc-inducible ESC lines from parental MGZ5TcH2 cells was as described (Masui et al., 2005; Mishima et al., 2007). HUVECs were obtained from Sanko Junyaku and cultured as described (Mishima et al., 2007). VEGF (R&D, 30 ng/ml), TGFβ1, 2 and 3 (R&D, 1 ng/ml), BMP4 (R&D, 50 ng/ml), BMP7 (R&D, 500 ng/ml), activin (R&D, 25 ng/ml), TβR-I inhibitor (Calbiochem LY364947, 1 μM) and tetracycline (Sigma-Aldrich, 1 μg/ml) were used.

RNA interference and oligonucleotides

siRNAs were introduced into cells as described (Shirakihara et al., 2007). The target sequences for mouse Snail and Smad4 siRNAs were 5′-UGCAGUUGAAGAUCUUCCGCGACUG-3′ and 5′-UUAAUCCUGAGAGAUCAAUUCCAGGS-3′, respectively. Control siRNAs were obtained from Ambion.

Immunohistochemistry and immunoblot analysis

Immunohistochemistry of cultured cells was performed as described (Yamashita et al., 2000) using monoclonal antibodies to PECAM1 (Mec13.3, BD Pharmingen), SMA (1A4, Sigma-Aldrich) and FLAG (M2, Sigma-Aldrich). Stained cells were photographed using a confocal microscope (LSM510 META, Carl Zeiss MicroImaging) with 10× (Plan-Neofluar 10×/0.30) objectives and LSM Image Browser. All images were taken at room temperature, and imported into Adobe Photoshop as TIFs for contrast adjustment and figure assembly. Immunoblot analyses were performed as described (Kawabata et al., 1998) using antibodies to claudin 5 (Zymed), SMA (Sigma-Aldrich), α-tubulin (Sigma-Aldrich), Snail (Cell Signaling) and E-cadherin (BD Transduction Laboratories).

RNA isolation and RT-PCR

Total RNA was prepared using RNeasy Reagent (Qiagen) and reverse-transcribed by random priming and using a Superscript First-Strand Synthesis Kit (Invitrogen). Quantitative RT-PCR analysis was performed using the GeneAmp 5700 Sequence Detection System (Applied Biosystems). All expression data were normalized to those for β-actin. For primer sequences, see supplementary material Table S1.

Chromatin immunoprecipitation (ChIP) assay

Endothelial cells derived from Tc-Snail ESCs were obtained in the absence or presence of Tc, and were incubated with or without TGFβ for 3 hours. Cells were fixed by adding formaldehyde and harvested. ChIP assays were carried out as described (Nishimura et al., 2006). In order to precipitate Smad3 and FLAG-tagged Snail, anti-Smad3 antibody (Upstate Biotechnology) and anti-FLAG (M2) antibody were used. PCR of the SMA promoter around the TGFβ hypersensitivity region was performed using immunoprecipitated chromatin with primers 5′-CAGTTGTTCTGAGGGCTTAGGATGTTTATC-3′ and 5′-ACAAGGAGCAAAGACGGGCTGAAGCTGGCC-3′.

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References

Arciniegas, E., Sutton, A. B., Allen, T. D. and Schor, A. M. (1992). Transforming growth factor β1 promotes the differentiation of endothelial cells into smooth muscle-like cells in vitro. *J. Cell Sci.* **103**, 521-529.

Arciniegas, E., Ponce, L., Hartt, Y., Graterol, A. and Carlini, R. G. (2000). Intimal thickening involves transdifferentiation of embryonic endothelial cells. *Anat. Rec.* **258**, 47-57.

Bartram, U., Molin, D. G., Wisse, L. J., Mohamad, A., Sanford, L. P., Doetschman, T., Speer, C. P., Poelmann, R. E. and Gittenberger-de Groot, A. C. (2001). Double-outlet right ventricle and overriding tricuspid valve reflect disturbances of looping, myocardialization, endocardial cushion differentiation, and apoptosis in TGF-β2-knockout mice. *Circulation* **103**, 2745-2752.

Batlle, E., Sancho, E., Franci, C., Dominguez, D., Monfar, M., Baulida, J. and Garcia De Herreros, A. (2000). The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat. Cell Biol.* **2**, 84-89.

Camenisch, T. D., Molin, D. G., Person, A., Runyan, R. B., Gittenberger-de Groot, A. C., McDonald, J. A. and Klewer, S. E. (2002). Temporal and distinct TGFβ ligand requirements during mouse and avian endocardial cushion morphogenesis. *Dev. Biol.* **248**, 170-181.

Cano, A., Perez-Moreno, M. A., Rodrigo, I., Locascio, A., Blanco, M. J., del Barrio, M. G., Portillo, F. and Nieto, M. A. (2000). The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat. Cell Biol.* **2**, 76-83.

Carmeliet, P. (2005). Angiogenesis in life, disease and medicine. *Nature* **438**, 932-936.

Carver, E. A., Jiang, R., Lan, Y., Oram, K. F. and Gridley, T. (2001). The mouse snail gene encodes a key regulator of the epithelial-mesenchymal transition. *Mol. Cell. Biol.* **21**, 8184-8188.

Coultas, L., Chawengsaksophak, K. and Rossant, J. (2005). Endothelial cells and VEGF in vascular development. *Nature* **438**, 937-945.

David, L., Mallet, C., Mazerbourg, S., Feige, J. J. and Bailly, S. (2007). Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. *Blood* **109**, 1953-1961.

DeRuiter, M. C., Poelmann, R. E., VanMunsteren, J. C., Mironov, V., Markwald, R. R. and Gittenberger-de Groot, A. C. (1997). Embryonic endothelial cells transdifferentiate into mesenchymal cells expressing smooth muscle actins in vivo and in vitro. *Circ. Res.* **80**, 444-451.

Derynck, R. and Zhang, Y. E. (2003). Smad-dependent and Smad-independent pathways in TGF-β family signalling. *Nature* **425**, 577-584.

Ema, M., Faloon, P., Zhang, W. J., Hirashima, M., Reid, T., Stanford, W. L., Orkin, S., Choi, K. and Rossant, J. (2003). Combinatorial effects of Flk1 and Tal1 on vascular and hematopoietic development in the mouse. *Genes Dev.* **17**, 380-393.

Feng, X. H. and Derynck, R. (2005). Specificity and versatility in TGF-β signaling through Smads. *Annu. Rev. Cell Dev. Biol.* **21**, 659-693.

Frid, M. G., Kale, V. A. and Stenmark, K. R. (2002). Mature vascular endothelium can give rise to smooth muscle cells via endothelial-mesenchymal transdifferentiation: in vitro analysis. *Circ. Res.* **90**, 1189-1196.

Goumans, M. J. and Mummery, C. (2000). Functional analysis of the TGF-β receptor/Smad pathway through gene ablation in mice. *Int. J. Dev. Biol.* **44**, 253-265.

Hirschi, K. K. and Majesky, M. W. (2004). Smooth muscle stem cells. *Anat. Rec.* **276**, 22-33.

Huber, M. A., Kraut, N. and Beug, H. (2005). Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr. Opin. Cell Biol.* **17**, 548-558.

Ishisaki, A., Hayashi, H., Li, A. J. and Imamura, T. (2003). Human umbilical vein endothelium-derived cells retain potential to differentiate into smooth muscle-like cells. *J. Biol. Chem.* **278**, 1303-1309.

Kawabata, M., Inoue, H., Hanyu, A., Imamura, T. and Miyazono, K. (1998). Smad proteins exist as monomers in vivo and undergo homo- and hetero-oligomerization upon activation by serine/threonine kinase receptors. *EMBO J.* **17**, 4056-4065.

Lee, J. M., Dedhar, S., Kalluri, R. and Thompson, E. W. (2006). The epithelial-mesenchymal transition: new insights in signaling, development, and disease. *J. Cell Biol.* **172**, 973-981.

Liebner, S., Cattelino, A., Gallini, R., Rudini, N., Iurlaro, M., Piccolo, S. and Dejana, E. (2004). β-catenin is required for endothelial-mesenchymal transformation during heart cushion development in the mouse. *J. Cell Biol.* **166**, 359-367.

Ma, L., Lu, M. F., Schwartz, R. J. and Martin, J. F. (2005). Bmp2 is essential for cardiac cushion epithelial-mesenchymal transition and myocardial patterning. *Development* **132**, 5601-5611.

Marchetti, S., Gimond, C., Iljin, K., Bourcier, C., Alitalo, K., Pouyssegur, J. and Pages, G. (2002). Endothelial cells genetically selected from differentiating mouse embryonic stem cells incorporate at sites of neovascularization in vivo. *J. Cell Sci.* **115**, 2075-2085.

Markwald, R. R., Fitzharris, T. P. and Manasek, F. J. (1977). Structural development of endocardial cushions. *Am. J. Anat.* **148**, 85-119.

Martinez-Estrada, O. M., Culleres, A., Soriano, F. X., Peinado, H., Bolos, V., Martinez, F. O., Reina, M. A., Fabre, M. and Vilaro, S. (2006). The transcription factors Slug and Snail act as repressors of Claudin-1 expression in epithelial cells. *Biochem. J.* **394**, 449-457.

Masui, S., Shimosato, D., Toyooka, Y., Yagi, R., Takahashi, K. and Niwa, H. (2005). An efficient system to establish multiple embryonic stem cell lines carrying an inducible expression unit. *Nucleic Acids Res.* **33**, e43.

Mercado-Pimentel, M. E. and Runyan, R. B. (2007). Multiple transforming growth factor-β isoforms and receptors function during epithelial-mesenchymal cell transformation in the embryonic heart. *Cells Tissues Organs* **185**, 146-156.

Mishima, K., Watabe, T., Saito, A., Yoshimatsu, Y., Imaizumi, N., Masui, S., Hirashima, M., Morisada, T., Oike, Y., Araie, M. et al. (2007). Prox1 induces lymphatic endothelial differentiation via integrin α9 and other signaling cascades. *Mol. Biol. Cell* **18**, 1421-1429.

Nishimura, G., Manabe, I., Tsushima, K., Fujiu, K., Oishi, Y., Imai, Y., Maemura, K., Miyagishi, M., Higashi, Y., Kondoh, H. et al. (2006). δEF1 mediates TGF-β signaling in vascular smooth muscle cell differentiation. *Dev. Cell* **11**, 93-104.

Oh, S. P., Seki, T., Goss, K. A., Imamura, T., Yi, Y., Donahoe, P. K., Li, L., Miyazono, K., ten Dijke, P., Kim, S. et al. (2000). Activin receptor-like kinase 1 modulates transforming growth factor-β1 signaling in the regulation of angiogenesis. *Proc. Natl. Acad. Sci. USA* **97**, 2626-2631.

Paranya, G., Vineberg, S., Dvorin, E., Kaushal, S., Roth, S. J., Rabkin, E., Schoen, F. J. and Bischoff, J. (2001). Aortic valve endothelial cells undergo transforming growth factor-β-mediated and non-transforming growth factor-β-mediated transdifferentiation *in vitro*. *Am. J. Pathol.* **159**, 1335-1343.

Peinado, H., Quintanilla, M. and Cano, A. (2003). Transforming growth factor β-1 induces snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions. *J. Biol. Chem.* **278**, 21113-21123.

Peinado, H., Olmeda, D. and Cano, A. (2007). Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat. Rev. Cancer* **7**, 415-428.

Potts, J. D., Dagle, J. M., Walder, J. A., Weeks, D. L. and Runyan, R. B. (1991). Epithelial-mesenchymal transformation of embryonic cardiac endothelial cells is inhibited by a modified antisense oligodeoxynucleotide to transforming growth factor β3. *Proc. Natl. Acad. Sci. USA* **88**, 1516-1520.

Sanford, L. P., Ormsby, I., Gittenberger-de Groot, A. C., Sariola, H., Friedman, R., Boivin, G. P., Cardell, E. L. and Doetschman, T. (1997). TGFβ2 knockout mice have multiple developmental defects that are non-overlapping with other TGFβ knockout phenotypes. *Development* **124**, 2659-2670.

Sawyer, J. S., Anderson, B. D., Beight, D. W., Campbell, R. M., Jones, M. L., Herron, D. K., Lampe, J. W., McCowan, J. R., McMillen, W. T., Mort, N. et al. (2003). Synthesis and activity of new aryl- and heteroaryl-substituted pyrazole inhibitors of the transforming growth factor-β type I receptor kinase domain. *J. Med. Chem.* **46**, 3953-3956.

Scharpfenecker, M., van Dinther, M., Liu, Z., van Bezooijen, R. L., Zhao, Q., Pukac, L., Löwik, C. W. and ten Dijke, P. (2007). BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis. *J. Cell Sci.* **120**, 964-972.

Shah, N. M., Groves, A. K. and Anderson, D. J. (1996). Alternative neural crest cell fates are instructively promoted by TGFβ superfamily members. *Cell* **85**, 331-343.

Shirakihara, T., Saitoh, M. and Miyazono, K. (2007). Differential regulation of epithelial and mesenchymal markers by δEF1 proteins in epithelial mesenchymal transition induced by TGF-β. *Mol. Biol. Cell* **18**, 3533-3544.

Timmerman, L. A., Grego-Bessa, J., Raya, A., Bertran, E., Perez-Pomares, J. M., Diez, J., Aranda, S., Palomo, S., McCormick, F., Izpisua-Belmonte, J. C. et al. (2004). Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. *Genes Dev.* **18**, 99-115.

Ullmann, U., In't Veld, P., Gilles, C., Sermon, K., De Rycke, M., Van de Velde, H., Van Steirteghem, A. and Liebaers, I. (2007). Epithelial-mesenchymal transition process in human embryonic stem cells cultured in feeder-free conditions. *Mol. Hum. Reprod.* **13**, 21-32.

Watabe, T., Nishihara, A., Mishima, K., Yamashita, J., Shimizu, K., Miyazawa, K., Nishikawa, S. and Miyazono, K. (2003). TGF-β receptor kinase inhibitor enhances growth and integrity of embryonic stem cell-derived endothelial cells. *J. Cell Biol.* **163**, 1303-1311.

Yamashita, J., Itoh, H., Hirashima, M., Ogawa, M., Nishikawa, S., Yurugi, T., Naito, M., Nakao, K. and Nishikawa, S. (2000). Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature* **408**, 92-96.

Zeisberg, E. M., Tarnavski, O., Zeisberg, M., Dorfman, A. L., McMullen, J. R., Gustafsson, E., Chandraker, A., Yuan, X., Pu, W. T., Roberts, A. B. et al. (2007a). Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. *Nat. Med.* **13**, 952-961.

Zeisberg, E. M., Potenta, S., Xie, L., Zeisberg, M. and Kalluri, R. (2007b). Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts. *Cancer Res.* **67**, 10123-10128.

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Development of stabilin2$^+$ endothelial cells from mouse embryonic stem cells by inhibition of TGFβ/activin signaling

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ABSTRACT

To understand the endothelial cell (EC) development, arterial, venous, and lymphatic EC (LEC) have been successfully induced from embryonic stem cells (ESC). However, tissue-specific EC, such as hepatic sinusoidal EC (HSEC), have never been generated from ESC. Based on the findings that TGFβ/activin signaling negatively regulates differentiation of both LEC and HSEC, and that HSEC and LEC are distinguishable by the expression of marker genes, we assessed the role of TGFβ/activin signaling in EC development from ESC. Here we show that the inhibition of TGFβ/activin signaling by a TGFβ receptor I (TGFβRI) kinase inhibitor increased the expression of Lyve1 and stabilin2 but not podoplanin in CD31$^+$CD34$^+$ EC derived from ESC. EC generated by the inhibition of TGFβRI signaling also exhibited stronger endocytic activity than control EC, indicating that their phenotype is similar to fetal HSEC. Our results reveal that TGFβ/activin signaling negatively regulates the early events of HSEC differentiation.

Endothelial cells (EC) are actively involved in various biological processes in different tissues, such as organ development, homeostasis, regeneration and disease [1], and their characteristics are differentially regulated in each vascular bed [2]. Progress has been made to distinguish various types of EC by expression of molecular markers, allowing us to address the development of EC heterogeneity [3]. Lymphatic endothelial cells (LEC) are EC that line the lymphatic vasculature. Because lymphatic vessels are important not only for the maintenance of fluid balance, but also for the pathogenesis of diseases, such as cancer [4], much attention has been paid to lymphatic development [5]. Hepatic sinusoidal EC (HSEC) are EC that line the hepatic microvasculature, sinusoids. It has been well known that HSEC have unique structural and functional characteristics, such as fenestrae and the scavenging activity [6], but the mechanism of their development remains largely unknown. Interestingly, it has been noted that there are some similarities between LEC and HSEC [7]. For example, both LEC and HSEC have minimal basement membranes and loose cell–cell junctions, express Lyve1, but lack CD34 [7]. Moreover, both EC are originated from veins or mesenchyme [8–10]. These observations suggest that the development of LEC and HSEC is regulated by the same signaling pathway to some extent.

We have recently described development of mouse HSEC by immunohistochemical studies [11]. Together with studies on rat and human HSEC [12–14], we have proposed that development of HSEC can be divided into at least three stages [11]. First, at the beginning of the liver specification, from embryonic day (E) E8.0 to E9.5 in mouse, EC in the emerging liver bud do not express an HSEC specific marker. At this stage, HSEC cannot be distinguished from other EC. At the second stage from E9.5 to perinatal period, HSEC acquire some of their specific features, such as the expression of stabilin2 (Stab2) and Lyve1 and high endocytic activity. At this stage, HSEC can be clearly distinguished from other EC based on such characteristics. However, their characteristics are different from adult HSEC. E14.5 HSEC express CD34 but lack the receptors for the Fc fragment of IgG (FcgRs) and fenestrae, whereas adult mature HSEC are CD34$^-$FcgRs$^+$ and possess fenestrae. It should be noted that the earliest marker of HSEC is Stab2 expression.

A recent report revealed that TGFβ/activin signaling inhibits the proliferation, migration and differentiation of LEC [15]. It has also been shown that TGFβ/activin signaling inhibits maturation of HSEC from the immature stage [16]. However, it still remains unknown what signals regulate the early stage of HSEC development.

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Embryonic stem cells (ESC) have been shown to differentiate into Flk1$^+$ vascular progenitors that give rise to both endothelial and mural cells [17]. This system has been considered to mimic the EC differentiation in vivo, and has been used to study the development of EC heterogeneity [18]. By using this differentiation system, we previously demonstrated that TGFβ/activin signaling inhibits proliferation and sheet formation of EC [19]. However, it remains unknown whether TGFβ/activin signaling also inhibits differentiation of LEC and/or HSEC in this system. In this study, we addressed this possibility by using the markers that distinguish LEC and HSEC from other EC [3,7,11]. Our results show that the inhibition of TGFβ/activin signaling in EC differentiated from ESC increases expression of Lyve1, a common marker for LEC and HSEC, but not podoplanin (Pdpn) and chemokine (C–C motif) ligand 21 (Ccl21), LEC markers. It also increases the expression of Stab2 and coagulation factor VIII (F8), and induces stronger endocytic activity. Our results suggest that TGFβ/activin signaling negatively regulates the early step of HSEC differentiation.

Materials and methods

Cell culture. Maintenance, differentiation and culture of CCE ESC (a gift from Sir Martin J. Evans, Cardiff University, UK) were performed as described previously [17,20]. Briefly, CCE ESC were maintained on gelatin-coated tissue culture dishes in KnockOut D-MEM (Invitrogen, California, USA) supplemented with 15% fetal bovine serum (FBS, SAFC Biosciences, Tokyo, Japan), 2000 U/mL LIF (Millipore, Billerica, USA), 5×10^{-5} M 2-mercaptoethanol (2-ME), L-glutamine and non-essential amino acids (Invitrogen). For differentiation, ESC were cultured 4 days on type-IV collagen-coated dishes (AGC TECHNO GLASS, Chiba, Japan) in differentiation medium (alpha minimal essential medium (Invitrogen) supplemented with 10% FBS (SAFC Biosciences) and 5×10^{-5} M 2-ME). Flk1$^+$ vascular progenitor cells were cultured in differentiation medium supplemented with 30 ng/mL recombinant human VEGF (R&D systems, Minneapolis, USA). To block TGFβ/activin signaling, 0.3 μM TGFβ Type I Receptor Kinase

Table 1
Oligonucleotides used in RT-PCR

Gene symbol	Description	GeneBank		Sequence
Actb	Actin, beta, cytoplasmic	NM_007393.1	Sense	5′-GAT ATC GCT GCG CTG GTC GTC-3′
			Antisense	5′-ACG CAG CTC ATT GTA GAA GGT GTG G-3′
Ccl21	Chemokine (C–C motif) ligand 21	NM_023052.1	Sense	5′-GAT GAT GAC TCT GAG CCT CC-3′
			Antisense	5′-CTC TTG AGG GCT GTG TCT GT-3′
F8	Coagulation factor VIII	NM_007977.1	Sense	5′-AAA GAA GGC AGT CTC TCC AAA-3′
			Antisense	5′-GGA ACT GCC CAA GAT CTA TCA-3′
Fcgr2b	Fc receptor, IgG, low affinity IIb	NM_001077189.1	Sense	5′-TGT GGA CAG CCG TGC TAA AT-3′
			Antisense	5′-CAG CAG CCA GTC AGA AAT CA-3′
Mrc1	Mannose receptor, C type 1	NM_008625	Sense	5′-ACC CTG TAT GCC TGT GAT TCG-3′
			Antisense	5′-AGG TGC AGT CTG CAT ACC ACT TGT-3′
Stab2	Stabilin-2	NM_138673.1	Sense	5′-GCA CCA CCT CAC TAA TGT CAA-3′
			Antisense	5′-CCC AAG AGG GTC ACT GTT CT-3′

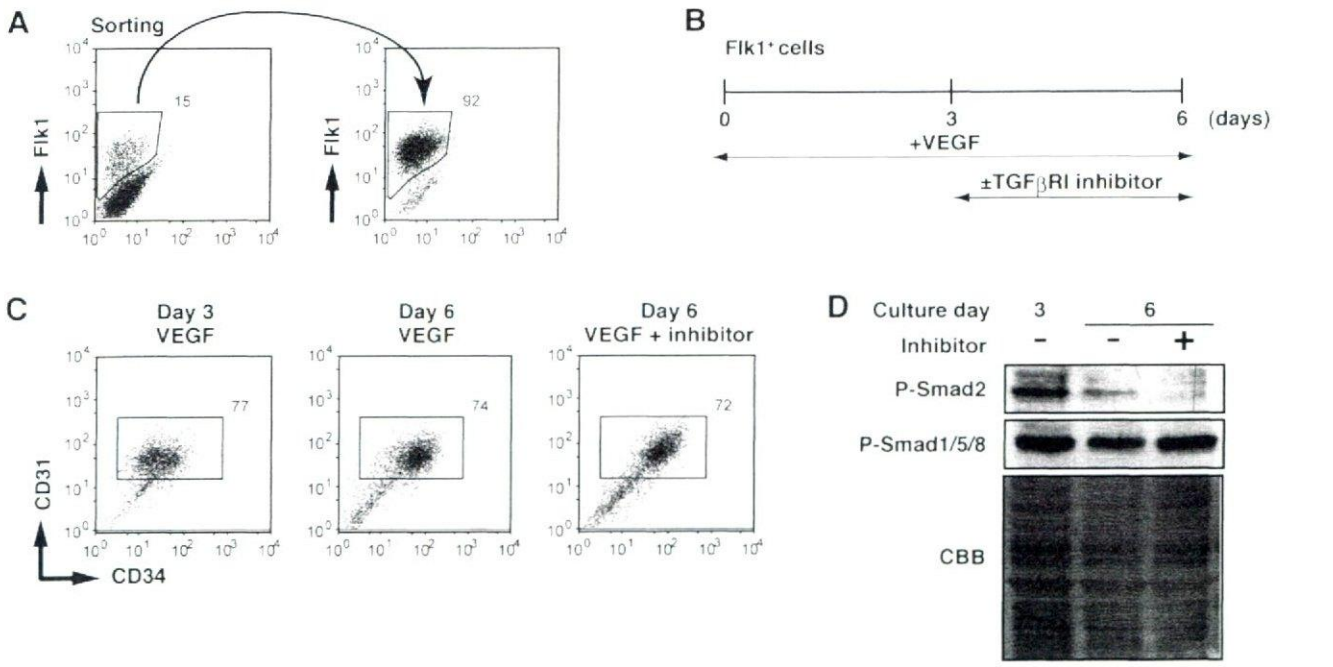

Fig. 1. Inhibition of TGFβRI signaling does not alter the differentiation of CD34$^+$CD31$^+$ cells from Flk1$^+$ vascular progenitors. (A) Isolation of Flk1$^+$ cells from ES-derived differentiated cells. ESC were cultured for 4 days. Flk1$^+$ cells were purified by using the autoMACS. The enclosures indicate Flk-1$^+$ cells. (B) Two protocols to culture the Flk1$^+$ vascular progenitors. (C) Expression of molecular markers for endothelial lineage, CD31 and CD34. Cells were harvested on culture day 3 and 6, and were subjected to flow cytometry. The enclosures indicate CD34$^+$CD31$^+$ cells. (D) Phosphorylation of Smad2 and Smad1/5/8 after cultivation of Flk1$^+$ cells. Cells were harvested on culture day 3 and 6. Cell lysates were directly subjected to immunoblotting using phospho-Smad2 and phosphor-Smad1/5/8 antibodies. Coomassie blue staining (CBB) was used as loading controls. The numbers in (A) and (C) indicate percentages of cells in each box. All results are representative of at least three independent experiments.

Inhibitor II (Tbr1ki2) (EMD Chemicals, Darmstadt, Germany) was added.

Cell preparation. Flk1$^+$ vascular progenitors were isolated from differentiated ESC after a 4-day culture as described previously [20]. Briefly, cells were trypsinized and suspended in staining buffer (phosphate-buffered saline (PBS) containing 0.5% BSA and 2 mM EDTA). Cells were stained with phycoerythrin (PE)-conjugated anti-mouse Flk1 monoclonal antibody (mAb) (eBioscience, San Diego, USA) followed by anti-PE microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells labeled with the microbeads were separated by using an autoMACS Separator (Miltenyi).

Flow cytometry. MAb against mouse CD16/32, CD31, CD34, and CD45 were purchased from BD Biosciences (San Jose, USA). MAb against mouse Stab2 and Lyve1 were established in our laboratory [11]. MAb against mouse Pdpn was kindly provided by Drs. Fujita and Tsuruo [21]. Cells were blocked with an anti-mouse CD16/32 mAb, co-stained with fluorescein- and biotin-conjugated antibodies, washed, and incubated with allophycocyanin-conjugated streptavidin (Invitrogen). Dead cells were stained with propidium iodide. Labeled cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences) and with FlowJo software (Tree Star, Inc., Ashland, USA).

Immunostaining, western blot analysis, and cellular uptake of scavenger ligands. Staining of cultured cells was performed as described previously [11]. Western blot analysis was performed as described previously [22]. Antibodies against phospho-Smad1/5/8 and phopho-Smad2 were purchased from Cell Signaling Technology (Danvers, USA). For cellular uptake of acetylated low density lipoprotein (Ac-LDL) and hyaluronan, cells were incubated with 5 μg/mL Ac-LDL labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine (DiI-Ac-LDL, Biomedical Technologies, Stoughton, USA) or 25 μg/mL fluoresceinamine labeled sodium hyaluronate (FITC-HA, PG Research, Tokyo, Japan) at 37 °C for 4 h, and counterstained with Hoechst dye. Images were captured and fluorescence intensity was quantified as described previously [11].

RT-PCR analysis. RNA was extracted using a High Pure RNA Isolation Kit (F. Hoffmann-La Roche, Basel, Switzerland). First-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) and random hexamer primers. The thermal cycle (denaturation at 94 °C for 15 s, annealing at an appropriate temperature for each pair of primers for 15 s and extension at 72 °C for 15 s) was repeated 35 times. The primers used are shown in Table 1.

Results

Induction of endothelial cells from ESC

To examine whether TGFβ/activin signaling inhibits differentiation of LEC and/or HSEC in the ESC differentiation system, EC were induced from Flk1$^+$ vascular progenitors derived from ESC (Fig. 1A–C) [17]. Flk1$^+$ vascular progenitors were isolated (Fig. 1A), and further cultured for 6 days in the presence of VEGF to induce EC differentiation (Fig. 1B). EC differentiation was assessed by the expression of pan-EC markers, CD31 and CD34. At the beginning of the culture, Flk1$^+$ cells did not express the pan-EC markers [17], however, on culture day 3, over 70% of cultured Flk1$^+$ cells expressed both CD31 and CD34, indicating that differentiation of EC had been induced at this period (Fig. 1C). To inhibit endogenous TGFβ/activin signaling in CD31$^+$CD34$^+$ EC, TGFβRI inhibitor (Tbr1ki2) was added to the cells on culture day 3 (Fig. 1B). On culture day 6, about 70% of the cells were CD31$^+$CD34$^+$ in the presence or absence of the Tbr1ki2 (Fig. 1C). Of note, both the percent-

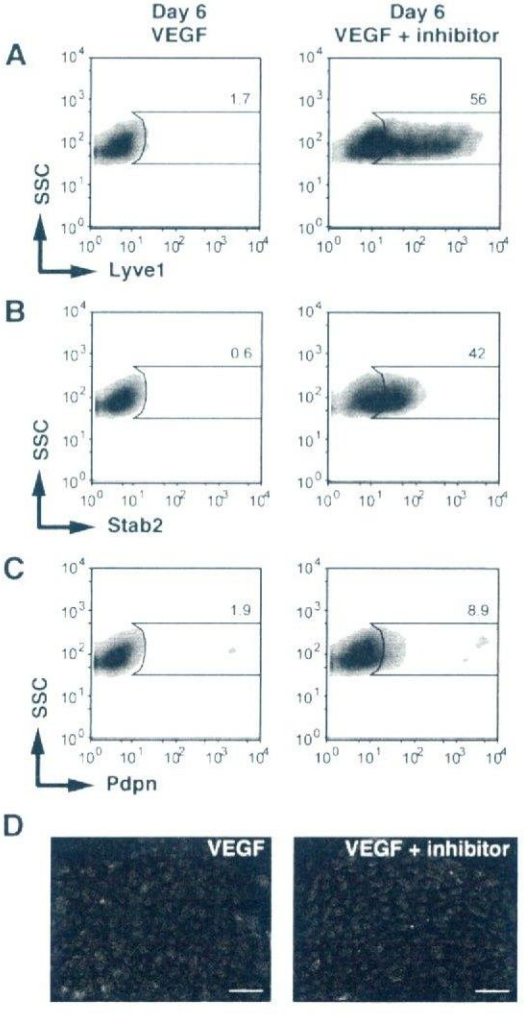

Fig. 2. Inhibition of TGFβRI signaling upregulates the expression of Lyve1 and Stab2, but not Pdpn. (A–C) Expression of molecular marker for HSEC and LEC. Flk1$^+$ cells were cultured for 6 days with or without TGFβRI inhibitor. On day 6, cells were harvested and were stained with antibodies against cell surface proteins as indicated. Shown are density plots of expression of Lyve1 (A), Stab2 (B) and Pdpn (C) in CD31$^+$CD34$^+$ cells. The numbers are percentages of the cells in each box. (D) Expression of Stab2 in the CD31$^+$ endothelial sheet. Flk1$^+$ cells were cultured for 6 days with or without the inhibitor and were stained with anti-CD31 (green), anti-Stab2 (magenta) antibodies and Hoechst dye (blue). All results are representative of at least three independent experiments. The bars represent 100 μm.

Fig. 3. Inhibition of TGFβRI signaling upregulates the expression of HSEC but not LEC markers. PCR analyses of genes for HSEC and LEC markers. Flk1$^+$ cells were cultured for 6 days with or without TGFβRI inhibitor (last 3 days). Cells were harvested on day 3, 4, 5 and 6, and were subjected to RT-PCR analyses. Positive and negative control templates were from blood vessels EC (BEC), LEC, and HSEC. All results are representative of at least three independent experiments.

ages and total numbers of the EC were not significantly different between VEGF alone and VEGF plus the inhibitor (Fig. 1C, Nonaka et al. unpublished observation).

To confirm the effect of Tbr1ki2, we examined phosphorylation of Smad2 and Smad1/5/8, which function downstream of the TGFβ/activins and bone morphogenetic proteins signaling pathways, respectively. On day 3, both Smad2 and Smad1/5/8 were phosphorylated by endogenous ligands (Fig. 1D), as we previously reported [19]. On day 6, the phosphorylation level of Smad2 was decreased in the absence of the inhibitor, and was hardly detectable in the presence of the inhibitor. However, the phosphorylation levels of Smad1/5/8 did not change between these conditions (Fig. 1D). These results indicate that TGFβ/activin signaling was actually blocked by Tbr1ki2 during the 3- to 6-day culture period.

Cell surface phenotype in EC developed with the TGFβRI inhibitor from ESC

To reveal the phenotype of EC derived from ESC, the expression of cell surface markers was examined in $CD31^+CD34^+$ cells (boxes in Fig. 1C) by flow cytometry. Lyve1 expression was expected to increase, because the previous study showed that the inhibition of TGFβ/activin signaling induced it in human dermal LEC [15]. As expected, treatment of ESC-derived EC with Tbr1ki2 for 3 days upregulated Lyve1 expression in $CD31^+CD34^+$ EC. 56% of them were $Lyve1^+$ in the presence of the inhibitor, but only 1.7% in its absence (Fig. 2A). To reveal whether the increased expression of Lyve1 represented LEC and/or HSEC differentiation, we examined the expression of Pdpn and Stab2, an LEC and an HSEC specific marker, respectively [11]. In the absence of Tbr1ki2, $CD31^+CD34^+$ EC did not express both markers on culture day 6 (Fig. 2B and C, left). In the presence of the inhibitor, Pdpn expression remained unchanged (Fig. 2C), however, Stab2 expression was increased in over 40% of $CD31^+CD34^+$ cells (Fig. 2B). The expression of Stab2 in EC was further confirmed by immunocytochemistry. As shown in Fig. 2D, Stab2 expression was detected on the $CD31^+$ EC sheet formed in the presence of Tbr1ki2, but not in the absence of it. These results indicate that the inhibition of TGFβRI signaling promotes differentiation of HSEC rather than LEC.

Gene expression profile of EC generated from ESC by the TGFβRI inhibitor

To further characterize the nature of the EC developed in the presence or absence of Tbr1ki2, we examined the expression of other LEC/HSEC markers by RT-PCR. As shown in Fig. 3, transcripts for HSEC markers, Stab2, F8, Fcgr2b and mannose receptor, C type 1 (Mrc1), were upregulated by the addition of Tbr1ki2. On the other hand, the expression of Ccl21, an LEC marker, was not detected regardless of the culture conditions. The upregulation of HSEC markers was observed on one day after the addition of Tbr1ki2, though the number of $Stab2^+$ EC and the expression level of Stab2 protein were more prominent on three days after the addition of the inhibitor (Nonaka et al. unpublished observation). These results further confirm differentiation of HSEC by the inhibition of TGFβRI signaling, and indicate that the induction begins just after the addition of Tbr1ki2.

Functional properties of EC generated from ESC by the TGFβRI inhibitor

To evaluate the functional properties of EC developed with Tbr1ki2, we examined the cellular uptake of fluorescently labeled Ac-LDL and hyaluronan. The incorporation of Ac-LDL is a common characteristic of EC. We previously showed that mouse E14.5 HSEC exhibited 2–3 times higher endocytic activity for Ac-LDL than E14.5 $Stab2^-Lyve1^-$ EC [11]. Furthermore, uptake of hyaluronan is a characteristic feature of HSEC [23]. As shown in Fig. 4A and B, EC developed with or without Tbr1ki2 incorporated Ac-LDL, while quantitative analysis revealed that those developed with Tbr1ki2 exhibited a higher (>3-fold) endocytic activity than those without the inhibitor (Fig. 4C). Moreover, EC developed with the inhibitor incorporated hyaluronan, while those without the inhibitor failed to do so (Fig. 4D and E). These results indicate that EC developed with Tbr1ki2 exhibit functional similarity with HSEC.

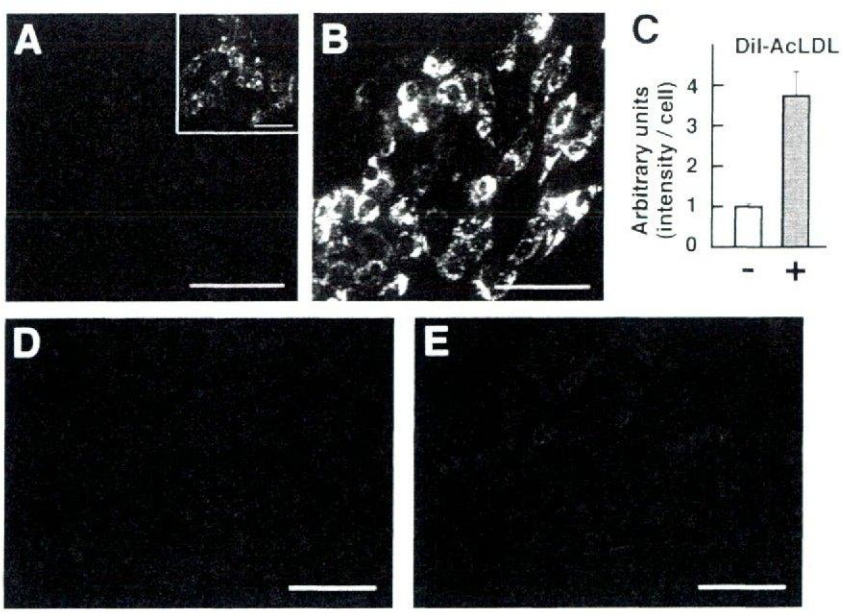

Fig. 4. EC with TGFβRI inhibitor exhibit higher endocytic activities. Endocytic activities in cultured EC differentiated from ESC. $Flk1^+$ cells were cultured for 6 days with (B,E) or without (A,D) the inhibitor, and were incubated with 5 μg/mL DiI-AcLDL (A,B) or 25 μg/mL FITC-hyaluronan (D,E) at 37 °C for 4 h. Cells were counterstained with Hoechst dye and were observed by fluorescence microscopy. The DiI fluorescence intensity was measured to quantify DiI-Ac-LDL uptake (C). Bars express means ± SD from three different microscopic fields. All results are representative of at least three independent experiments. The inset in A is an image of the same field taken by longer exposure (over 4 times long). The bars in (A,B) and (D,E) represent 80 and 100 μm, respectively.